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13. ABSTRACT (Maximum 200 Words) Degradation of the basement membrane (BM) surrounding breast epithelial units (acini) is associated with tumor progression. It is crucial to understand the molecular mechanisms that underlie the maintenance of an intact BM in order to develop anti-cancer strategies. Using non-neoplastic human breast epithelial S1 cells that differentiate into acini in the presence of extracellular matrix, we have shown a link between the nuclear organization of the protein NuMA and the maintenance of acinar differentiation, notably BM integrity. Sequence analysis of the distal portion of NuMA C-terminus (NuMA-CTDP) revealed that this region may be restricted to vertebrates and may adopt a structure possessing organizational and signaling properties. In addition, NuMA-CTDP shares similarities with sequences of proteins involved in chromatin structure. Expression of NuMA-CTDP in S1 cells altered chromatin structure and impaired acinar differentiation and formation of the BM. Cell fractionation showed that NuMA interacts with the chromatin compartment and suggested that NuMA might be associated with multi-protein complexes. NuMA has been recently identified as a possible candidate for breast cancer predisposition. By establishing a role for NuMA in chromatin organization critical for mammary epithelial differentiation, our work sheds light on how alterations in NuMA function may lead to cancer development.				
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INTRODUCTION

Differentiation of functional and structural epithelial units of breast tissue, referred to as acini, is maintained by the presence of a continuous basement membrane (BM). The breakdown of the BM by matrix proteases has been associated with loss of structural and functional differentiation and tumor progression. Understanding how an intact BM is maintained in normal epithelial structures should help develop new therapeutic tools to prevent cancer progression. We have reported a link between the organization of the nuclear mitotic apparatus protein NuMA in the cell nucleus, and the activation of matrix proteases and subsequent degradation of the BM (Lelièvre et al., 1998). We have hypothesized that the nuclear organization of NuMA induced by acini is contingent upon NuMA binding to other proteins to form multicomplexes, and that in turn, the supramolecular organization of these multicomplexes is critical for the acinar differentiation stage. In order to decipher the molecular mechanisms that link NuMA organization to the maintenance of acinar differentiation we have proposed to investigate the role of specific sequences of NuMA that we have identified as potential mediators of NuMA functions in the cell nucleus and explore the subcompartmentalization of NuMA.

BODY

Our working model is a non-neoplastic human mammary epithelial cell line HMT-3522 (S1) that can be induced to form functional acini surrounded by a complete endogenous BM when cultured in the presence of an exogenous extracellular matrix (MatrigelTM, Becton Dickinson) for 10 days (Petersen et al., 1992). *Using this system we have investigated the role of NuMA sequences as potential effectors of NuMA functions in the establishment and maintenance of acinar differentiation (statement of work; task 1), searched for potential binding partners of these sequences (statement of work; tasks 2 and 3), and analyzed the dynamics of NuMA compartmentalization (task 4).*

Task 1 (completed): Production and analysis of acini expressing truncated forms of NuMA.

Data obtained upon accomplishing this task have been combined into a manuscript that will be submitted shortly. See experimental results below.

We first confirmed that NuMA is abundantly expressed in human adult mammary epithelial tissue showing different levels of activity (i.e., resting vs lactating) (Figure 1 A-B). Using fluorescence immunostaining on sections of paraffin embedded tissue, we also confirmed that NuMA distribution usually appears as foci concentrated in the mid-nucleus region of differentiated mammary epithelial cells (Figure 1 C).

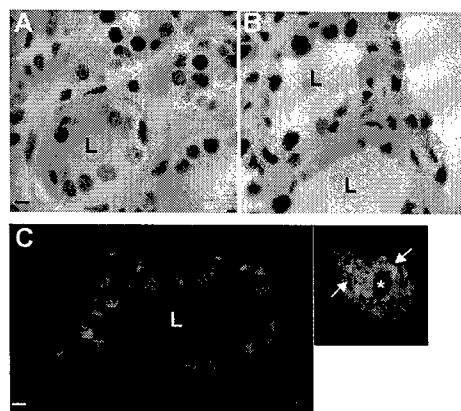


Figure 1. NuMA expression in human breast epithelium. A, B, C, immunostaining for NuMA on paraffin sections of human adult mammary gland. A-B, hrp-stained NuMA (brown) in resting (A) and lactating (B) mammary gland. Tissue is counterstained with hematoxylin. C, Fluorescently stained NuMA in the resting mammary gland (green). Inset shows one nucleus with abundance of NuMA foci in the mid-nucleus area (see arrows) and around the nucleolus (*). Size bar= 5 microns. L= lumen.

A fraction of NuMA is contained in the chromatin compartment in interphase.

The cell nucleus can be subdivided into several compartments including the soluble compartment in which components are easily extractable by detergents, the chromatin compartment in which components are released upon DNA digestion, and the insoluble compartment or nuclear matrix in which components are resistant to detergent extraction as well as DNA digestion. NuMA has been generally considered as a nuclear matrix protein based on its presence in fractions resistant to detergent extraction and DNA digestion (Lydersen and Pettijohn, 1980; Zeng *et al.*, 1994). However, there is evidence that NuMA may co-localize and/or interact with the non-histone protein HMG I/Y (Tabellini *et al.*, 2001), putatively involved in the regulation of gene expression, and the alleged transcription factor GAS41 (Harborth *et al.*, 2000), raising the possibility that NuMA may not only be part of the nuclear matrix but might also be linked to the chromatin compartment. In light of this possibility, we methodically assessed the nuclear compartmentalization of NuMA in the HMT-3522 breast model using two different methods of nuclear fractionation, and *in situ* degradation of DNA.

Non-neoplastic HMT-3522 S1 HMECs (Briand *et al.*, 1987) were cultured as monolayers (2D culture) to produce a high number of non-differentiated cells, and nuclear matrix fractions were prepared according to classical protocols (Nickerson *et al.*, 1997). In this method, after detergent extraction, nuclei are subjected to DNase I digestion, then nuclear matrix components are obtained by centrifugation following treatment with 2 M NaCl. The supernatant obtained following DNase I digestion and the pellet obtained after

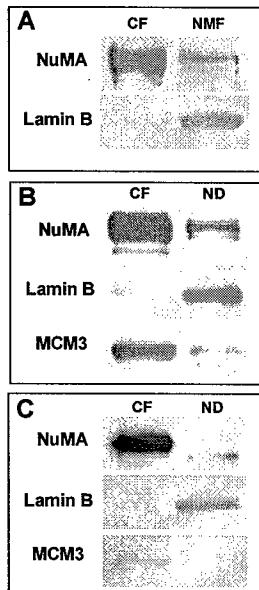


Figure 2. NuMA is associated with the chromatin compartment. A, B, C, Western blot for NuMA, Lamin B, and MCM3. A, S1 cells were cultured as a monolayer (2D) for ten days. Cells were fractionated using a classical protocol to obtain nuclear matrices, including treatment with 130 µg/ml DNase I for 30 minutes. B-C, S1 cells were cultured as a monolayer (2D) (B) or in 3D (C) for 10 days, and fractionated using a classical protocol for chromatin isolation, including 5 minutes incubation with 1 unit of micrococcal nuclease. CF: chromatin fraction; NMF: nuclear matrix fraction; ND: non-digestible nuclear fraction.

incubation with 2 M NaCl correspond to the chromatin fraction and the nuclear matrix fraction, respectively. Western blot analysis revealed that, as expected, NuMA was present in the nuclear matrix fraction. However, a high amount of NuMA was also found in the chromatin fraction (Figure 2A). Lamin B, a nuclear matrix protein considered the cornerstone of the insoluble nuclear shell, was predominantly found in the nuclear matrix fraction and hardly detected in the chromatin fraction (Figure 2A). To confirm that

NuMA was present in the chromatin fraction, nuclei were isolated from cells cultured under 2D conditions and treated to separate the chromatin from the non-digestible nuclear remnant according to classical chromatin fractionation methods (Wysocka *et al.*, 2001). Western blot analysis indicated that NuMA was present in the non-digestible nuclear compartment obtained upon micrococcal nuclease treatment, in agreement with its status of protein resistant to extraction; however, NuMA was also highly present in the chromatin compartment (Figure 2B). To verify that there had not been any significant cross-

contamination between fractions during the preparation, the same nitrocellulose membranes were blotted for chromatin-associated protein MCM3 (Takei *et al.*, 2001) and lamin B. MCM3 was enriched in the chromatin compartment, and lamin B was enriched in the non-digestible nuclear remnant, indicating that the fractionation had been successful (Figure 2B).

NuMA becomes distributed into distinct and large foci upon breast differentiation into acini (see figure 1C-inset and Lelièvre *et al.*, 1998), a process that is recapitulated upon culture of non-neoplastic S1 HMECs under 3D conditions in the presence of MatrigelTM. To assess whether NuMA was also present in the chromatin compartment in differentiated cells, chromatin fractions were prepared from cells organized into acini. Similarly to what was observed in 2D culture, NuMA was highly present in the chromatin fraction of acinar cells (Figure 2C).

NuMA distribution in mammary acinar cells is sensitive to DNA degradation and displays perinucleolar features.

A link between NuMA distribution and mammary epithelial differentiation was suggested previously by experiments showing the existence of a pattern of NuMA distribution specific for mammary acinar cells (Lelièvre *et al.*, 1998; Knowles *et al.*, submitted) in 3D culture and on biopsy sections of normal tissue. We reasoned that, since NuMA was found in the

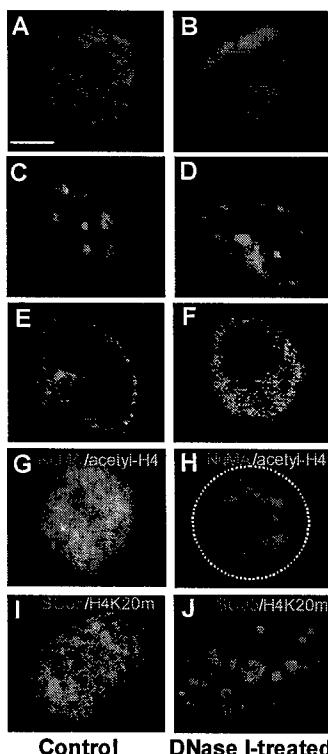


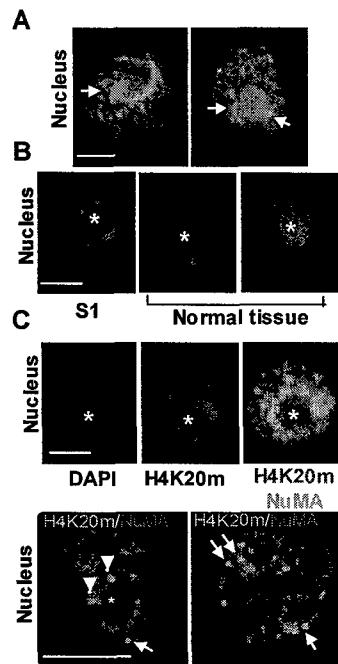
Figure 3. NuMA distribution is altered upon DNase I treatment. S1 cells were cultured in 3D for 10 days to induce acinar differentiation. **A-F**, Acinar cells were permeabilized with triton X-100 without DNase treatment (A, C, E) or treated with DNase I for 30 minutes (B, D, F) before fixation and immunostaining for NuMA (red, A-B), PML (green, C-D), and lamin B (green, E-F). **G-J**, Acinar cells were permeabilized with triton X-100 without DNase treatment (G, I) or treated with DNase I for 30 minutes (H, J) before fixation and dual immunostaining for NuMA (red) and acetyl-H4 (green) (G-H), and SC-35 (red) and H4K20m (green) (I-J). DAPI was used for DNA staining and is shown in images B-F and H-J. One nucleus is shown per image. Size bar= 2.5 microns.

chromatin fraction obtained upon DNase I treatment of differentiated HMECs (see figure 2C), part of the specific distribution of NuMA observed by immunostaining should be sensitive to DNA degradation. We incubated live cells organized into acini with DNase I according to previously utilized methods (Szekely *et al.*, 1999; Kaminker *et al.*, 2005). Immunostaining of NuMA in control (triton permeabilized only) and DNase I-treated 3D cultures revealed that a large portion of NuMA staining pattern was lost upon DNA digestion and that, in DNase I-treated cells, remaining NuMA

staining was located mainly at the nuclear perimeter and as a small circle within the cell nucleus (Figure 3 A-B). DNase I degrades easily accessible DNA first, hence there usually remain areas of non-digested DNA upon incomplete treatment with this enzyme. Dual staining for DNA and NuMA in DNase I-treated acinar cells showed that, in cases of incomplete degradation of DNA, NuMA staining was exactly delineating DNA staining

(Figure 3 B). However, a portion of NuMA staining remained upon complete degradation of DNA, which was consistent with a fraction of NuMA proteins being part of the nuclear matrix (see Figure 3 H). In contrast, PML, another coiled-coil protein found in nuclear matrix fractions, showed no dramatic alteration in its distribution upon DNase I treatment and no particular relationship with the remaining DNA (Figure 3 C-D). Staining for nuclear shell protein lamin B remained at the periphery of the nuclei upon DNase I treatment and was mostly delineating the outside of peripheral, nondigested DNA (Figure 3 E-F). To verify that DNase I treatment affected chromatin components, we analyzed the staining pattern of chromatin markers acetyl-H4 and H4K20m following DNase I treatment. Staining for both proteins was almost totally lost in the majority of cells treated with DNase I, whereas immunostaining for SC35, indicative of non-chromatin splicing speckles, appeared unaltered (Figure 3 G-J).

The formation of distinct foci of NuMA upon acinar differentiation is not seen usually throughout the cell nucleus, but rather concentrates in mid-



nuclear regions (see Figure 1 C-inset; Knowles *et al.*, submitted).

By comparing with DAPI staining, we observed that the mid-nuclear accumulation of NuMA foci usually corresponded to the perinucleolar region. The preeminence of NuMA around nucleoli in acinar cells was confirmed by dual staining for NuMA and nucleophosmin, a marker of nucleoli (Figure 4 A). Interestingly the perinucleolar region is a major center of higher order chromatin structure (i.e., the nuclear organization corresponding to the concentration of euchromatin and heterochromatin regions to specific locations in the cell nucleus). Classically, heterochromatin domains have been observed to concentrate at the nuclear periphery and around the nucleolus upon cellular differentiation (Chaly *et al.*, 1996; Olson 2002). Immunostaining for heterochromatin marker H4K20m in differentiated HMECs, both in 3D culture and on sections of normal breast tissue, indicated that the perinucleolar concentration of heterochromatin is also a trait of HMECs

Figure 4. NuMA is located in regions enriched in heterochromatin in acinar cells. S1 cells were cultured in 3D for 10 days to induce acinar differentiation. S1 acini and paraffin sections of archival biopsies of normal breast tissue were used for immunostaining. **A**, Immunostaining for NuMA (green) and nucleophosmin (red) in S1 acinar cells. Arrows indicate areas where NuMA concentrates around the nucleolus. **B**, Immunostaining for H4K20m (red) in S1 acinar cells [S1] and luminal cells from normal breast tissue [normal tissue]. Nuclei are counterstained with DAPI (blue). **C**, *Top panel*: Dual staining for H4K20m (red) and NuMA (green) in S1 acinar cells shows the concentration of H4K20m and NuMA domains around the nucleolus and some staining overlap (yellow). The nucleolus is identified by DAPI staining (blue). *Bottom panel*: higher magnification of dual staining for NuMA (red) and H4K20m (green) indicates that domains formed by the two proteins often seem to intercalate around the nucleolus (see arrowheads). A few H4K20m domains co-localize with NuMA staining (as shown by the yellow color-see arrows). One nucleus is shown per image. Size bar= 2.5 microns. [*] indicates the position of the nucleolus.

differentiation (Figure 4 B). In addition, H4K20m foci often intercalated and sometimes overlapped with aggregates of NuMA found at the periphery of the nucleolus in acinar cells co-stained for NuMA and H4K20m (Figure 4 C). Thus, NuMA appears to be present in several nuclear compartments in differentiated breast epithelium, and part of its acinar differentiation-specific distribution outlines specific features of higher order chromatin organization.

The C-terminus of NuMA is critical for proper acinar differentiation.

Acinar differentiation corresponds to the formation of baso-apically polarized multicellular structures characterized by the presence of a basement membrane at the basal side of cells and a lumen at the apical side of cells (Petersen *et al.*, 1992). We have shown previously that introduction of antibodies directed against the C-terminus of NuMA in pre-formed acini modified the nuclear distribution of endogenous NuMA and induced the loss of acinar differentiation, notably by altering basal polarity, as shown by the lack of continuous staining for basement membrane component collagen IV around the acini (Lelièvre *et al.*, 1998). Interestingly, the alteration of NuMA organization was accompanied by changes in the distribution of chromatin marker acetyl-H4 (Lelièvre *et al.*, 1998), suggesting that altering NuMA function by acting at its C-terminus may affect chromatin structure. We looked for regions within the C-terminus of NuMA that could reflect a possible function at the level of chromatin. Notably we focused on the distal portion of the C-terminus (NuMA-CTDP), which we had previously identified as highly conserved in vertebrates (Abad *et al.*, 2004), thus suggesting a potentially important role for this region in NuMA function. Using NuMA (NP_006176) residues 1965-2101 as query for a BLASTp search against the NCBI nonredundant database, including 2,315,908 sequences of all organisms, we identified a 81 amino acid segment (residues 1988 to 2068) in the distal portion of NuMA C-terminus that shared similarities with the central portion (residues 327-407) of histone promoter control 2 protein (HPC2) (NP_009774). HPC2 is a yeast regulator of histone expression and chromatin structure (Xu *et al.*, 1992). The match between the C-terminal segment of NuMA and the central sequence of HPC2 was statistically significant with E-value 0.006.

We used the C-terminal NuMA residues 1965-2101 to engineer a peptide that would compete with the C-terminus of endogenous NuMA for binding properties. Two FLAG-tagged constructs coding for the distal C-terminal region were prepared: One construct codes for the full 1965-2101 peptide sequence, which includes the nuclear localization signal (NLS) located within the 1984-2005 region (Gueth-Hallonet *et al.*,

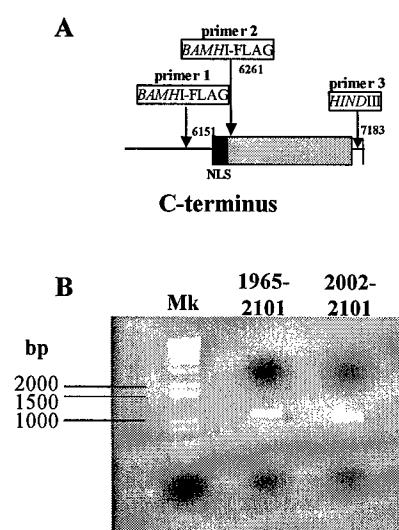


Figure 5. cDNA inserts for NuMA₁₉₆₅₋₂₁₀₁ and NuMA₂₀₀₂₋₂₁₀₁. **A**, Schematic of primers used to synthesize the cDNAs for NuMA₁₉₆₅₋₂₁₀₁ (primers 1 and 3) and NuMA₂₀₀₂₋₂₁₀₁ (primers 2 and 3) in relation to the position of the NLS. Numbers indicate the position of nucleotides. **B**, DNA electrophoresis of cDNA prepared from primers 1 and 3 (lane 1965-2101) and primers 2 and 3 (lane 2002-2101). Mk=marker; bp=base pair.

1996); the other construct codes for residues 2002-2101 that correspond to a sequence lacking the NLS (Figure 5).

Following transfection in S1 HMECs, expression of these constructs was verified by Western blot analysis from stable transfectants cultured under 2D conditions and/or FLAG immunostaining under 3D conditions (Figure 6 A-B). Ten clones of NuMA₁₉₆₅₋₂₁₀₁-S1 stable transfectants and three clones of NuMA₂₀₀₂₋₂₁₀₁-S1 stable transfectants were cultured under 3D conditions to induce the formation of acini. S1 cells were also transfected with insertless pCDNA 3.1 vector and five clones of insertless vector-S1 transfectants were cultured under 3D conditions. Immunostaining for FLAG in multicellular structures obtained under 3D culture conditions revealed that the location of FLAG-NuMA₁₉₆₅₋₂₁₀₁ peptide was strictly nuclear while the location of FLAG-NuMA₂₀₀₂₋₂₁₀₁ peptide was mainly cytoplasmic (Figure 6 B). Expression of the peptide was observed in 20% to 80% of cells depending on the clone (S1 cells easily shut off transgene expression). Only S1 cells transfected with insertless vector or NuMA₂₀₀₂₋₂₁₀₁ formed well-differentiated acini. In contrast there was a 20% to 80% decrease (corresponding to the extent of NuMA alteration in each clone) of well-formed acini in NuMA₁₉₆₅₋₂₁₀₁-transfectants, as shown by altered organization of alpha-6 integrin, beta-catenin, and collagen IV -all markers of the polarity axis- compared to the distribution observed in phenotypically normal epithelium (Figure 6 C-D). Immunostaining using two different antibodies that do not recognize the distal C-terminal region of NuMA showed that endogenous NuMA was diffusely distributed in 20% to 80% (corresponding to the extent of NuMA alteration in each clone) of the nuclei of acini formed by NuMA₁₉₆₅₋₂₁₀₁-S1 cells, whereas it was distributed into the foci-like pattern characteristic of acinar differentiation in insertless vector-S1 cells as well as in NuMA₂₀₀₂₋₂₁₀₁-S1 cells (Figure 6 E). To further investigate how the expression of NuMA₁₉₆₅₋₂₁₀₁ peptide had altered the organization of NuMA, we assessed whether the distribution of endogenous NuMA was still sensitive to DNA degradation. NuMA₁₉₆₅₋₂₁₀₁-S1, NuMA₂₀₀₂₋₂₁₀₁-S1, and insertless vector-S1 cells were cultured under 3D conditions for 10 days and subjected to DNase I digestion *in situ*. Immunostaining for endogenous NuMA revealed that, in contrast to NuMA₂₀₀₂₋₂₁₀₁-S1 cells and insertless vector-S1 cells, NuMA distribution was not affected by DNase I treatment in NuMA₁₉₆₅₋₂₁₀₁-S1 cells (Figure 6 F), indicating that the relationship between endogenous NuMA and chromatin has been altered upon expression of NuMA C-terminal peptide.

Alteration of the function of NuMA C-terminus (CT) is associated with changes in higher order chromatin structure characteristic of acinar differentiation.

To assess whether the expression of NuMA₁₉₆₅₋₂₁₀₁ was associated with changes in chromatin organization as previously observed upon introduction of antibodies against the C-terminus of NuMA (Lelièvre *et al.*, 1998), insertless vector-S1, NuMA₁₉₆₅₋₂₁₀₁-S1 and NuMA₂₀₀₂₋₂₁₀₁-S1 cells were immunostained for acetyl-H4 and heterochromatin marker H4K20m. Depending on the extent of expression of the peptide in the different clones, 20% to 80% of the nuclei in acini formed by NuMA₁₉₆₅₋₂₁₀₁-S1 cells had an altered organization of higher order chromatin structure, as shown by the accumulation of enlarged domains of acetyl-H4 and H4K20m at the nuclear periphery (Figure 7). In contrast, the distributions of acetyl-H4 and H4K20m in NuMA₂₀₀₂₋₂₁₀₁-S1 and insertless vector-S1 acinar cells were similar to those seen in non-transfected S1 cells. These data suggest that preventing the organization of NuMA into distinct nuclear foci characteristic of acinar differentiation by expressing the NuMA₁₉₆₅₋₂₁₀₁ peptide may, in turn, influence higher order chromatin organization associated with acinar differentiation.

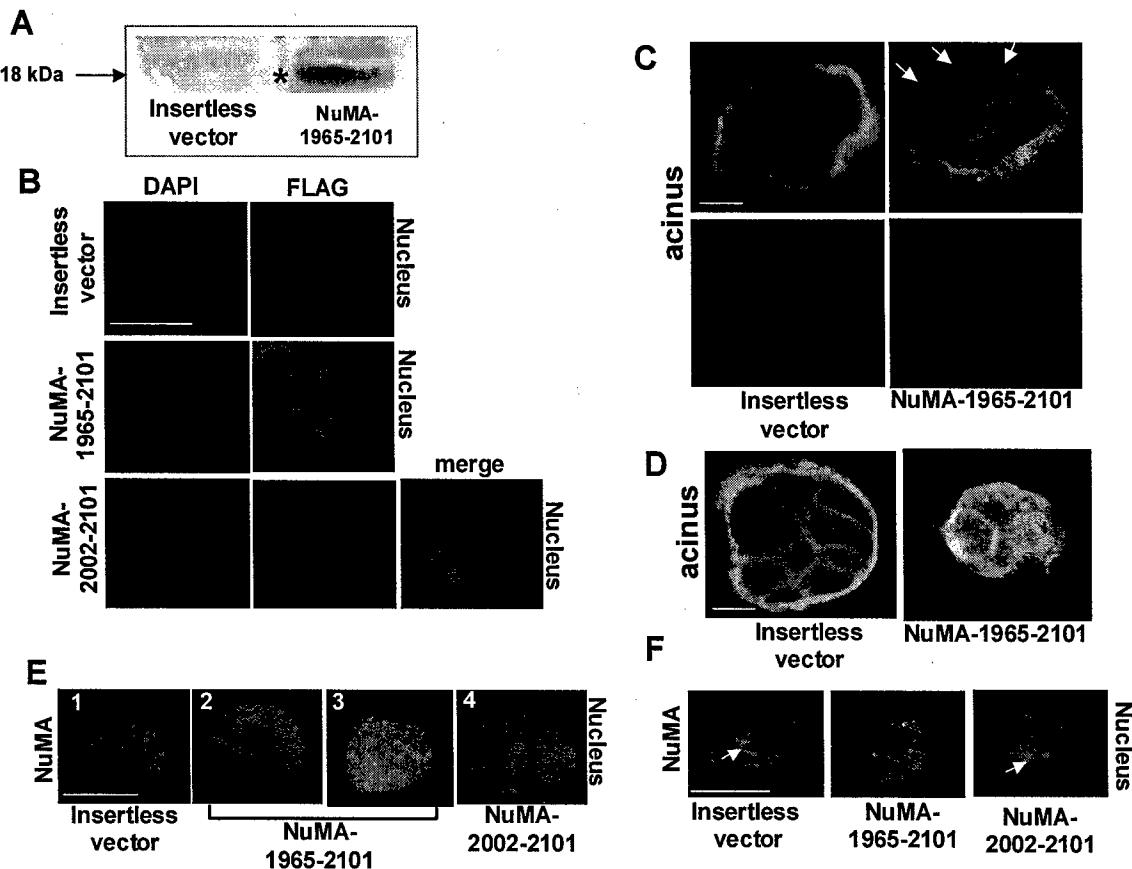


Figure 6. Cells expressing NuMA₁₉₆₅₋₂₁₀₁ display an altered organization of endogenous NuMA and lack of proper acinar differentiation. **A**, Western blot for 18 kDa FLAG-NuMA₁₉₆₅₋₂₁₀₁ peptide (*) in control (insertless vector) and NuMA₁₉₆₅₋₂₁₀₁-S1 cells cultured under 2D conditions. **B-E**, NuMA₁₉₆₅₋₂₁₀₁-S1, NuMA₂₀₀₂₋₂₁₀₁-S1, and insertless vector-S1 cells were cultured for 10 days under 3D conditions to induce acinar morphogenesis. **B**, Immunostaining for FLAG. Nuclei are counterstained with DAPI (blue). **C**, Dual immunostaining for FLAG (red) and collagen IV (green) shows alterations of basement membrane integrity (see arrows) in cells expressing NuMA₁₉₆₅₋₂₁₀₁ peptide. Nuclei are counterstained with DAPI (blue). **D**, Dual immunostaining for beta-catenin (red) and alpha 6-integrin (green) shows alterations in the internal organization of acinar cells in NuMA₁₉₆₅₋₂₁₀₁-S1 transfectants. The proper distribution of beta-catenin is characterized by its location only at plasma membranes involved in cell-cell adhesion, and the proper distribution of alpha 6-integrin is characterized by continuous staining at plasma membranes only involved in cell-ECM adhesion (compare proper distribution in [insertless vector] with altered distribution in [NuMA-1965-2101]). Nuclei are counterstained with DAPI (blue). **E**, Immunostaining for endogenous NuMA (red) in images 1, 2 and 4, and dual staining for NuMA (red) and FLAG (green) in image 3. **F**, NuMA₁₉₆₅₋₂₁₀₁-S1, NuMA₂₀₀₂₋₂₁₀₁-S1, and insertless vector-S1 cells were cultured for 10 days in 3D to induce acinar morphogenesis and incubated with or without DNase I prior to fixation. Shown is immunostaining for NuMA following DNase I treatment. The arrow indicates NuMA domains remaining towards the center of the cell nucleus. Size bar= 5 microns.

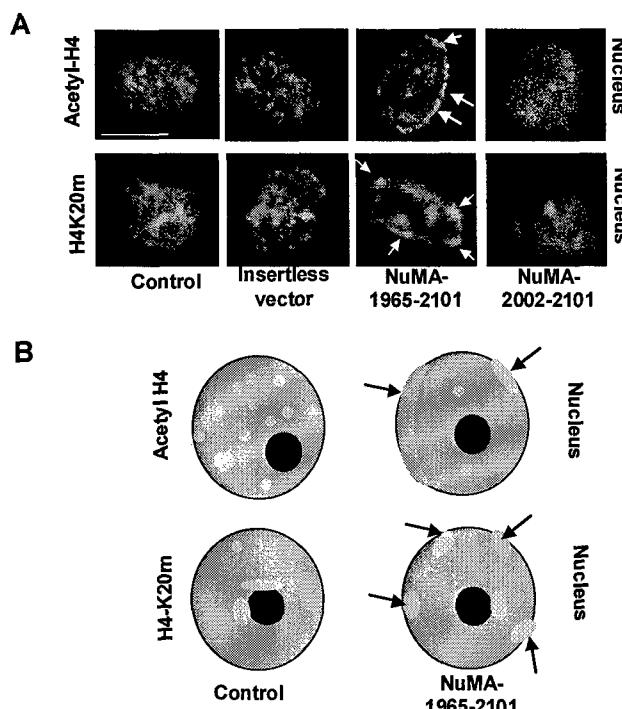


Figure 7. Alteration of NuMA function induces changes in higher order chromatin structure. **A,** NuMA₁₉₆₅₋₂₁₀₁-S1, NuMA₂₀₀₂₋₂₁₀₁-S1, insertless vector-S1, and control (nontransfected)-S1 cells were cultured for 10 days in 3D to induce acinar morphogenesis. Shown is immunostaining for chromatin marker acetyl-H4 and heterochromatin marker H4K20m. **B,** Diagrams outline the alterations in the higher order organization of acetyl-H4 and H4K20m commonly observed in NuMA₁₉₆₅₋₂₁₀₁-S1 cells. Dark green represents the diffuse staining for these proteins and light green represents the concentration of acetyl-H4 and H4K20m to specific areas of the cell nucleus. Arrows indicate acetyl-H4 and H4K20m domains mainly found at the nuclear periphery upon alterations of NuMA function. Size bar = 5 microns.

The changes observed in chromatin organization in NuMA₁₉₆₅₋₂₁₀₁-S1 cells could be a direct effect of the alteration of NuMA function. However, HMECs that express NuMA₁₉₆₅₋₂₁₀₁ show strong alterations in acinar differentiation; therefore, it is possible that the changes observed in chromatin organization in these cells are the result of signaling induced by the loss of basement membrane integrity (see figure 6 C). The peptides coding for NuMA-CT are expressed throughout acinar morphogenesis, which prevents us from easily assessing the relationship between the alteration of NuMA function, the inhibition of proper differentiation and the impairment of higher order chromatin reorganization. To evaluate whether altering NuMA function may influence higher order chromatin structure prior to impairing acinar differentiation, we treated preformed acini with function-blocking antibodies as done previously (Lelièvre *et al.*, 1998), but this time we fixed cells after 30 minutes of antibody incubation. After such a short incubation time, there were no alterations in acinar morphogenesis, as shown by the correct distribution of basement membrane component collagen IV (Figure 8 A-B). Nevertheless, the nuclei of anti-NuMA antibody treated cells already displayed a change in NuMA distribution and a peripheral concentration of H4K20m domains. In contrast, incubation with nonspecific immunoglobulins (IgGs) did not affect NuMA or H4K20m distribution (Figure 8 B). We also incubated S1 acinar cells with anti-NuMA antibodies or IgGs for three days, which is sufficient to induce changes in chromatin organization and the loss of basement membrane integrity (Lelièvre *et al.*, 1998). However, half of the cultures were also incubated with GM6001, an inhibitor of metalloproteinases that prevents the degradation of the basement membrane in anti-NuMA antibody-treated cells (Lelièvre *et al.*, 1998 and Figure 8 C). Acini cultured with or without GM6001 in the presence of NuMA antibodies had alterations in the distribution of acetyl-H4 and H4K20m (Figure 8 D). These data indicate that changes in higher order chromatin structure occur prior to alterations in basal polarity upon impairing NuMA function.

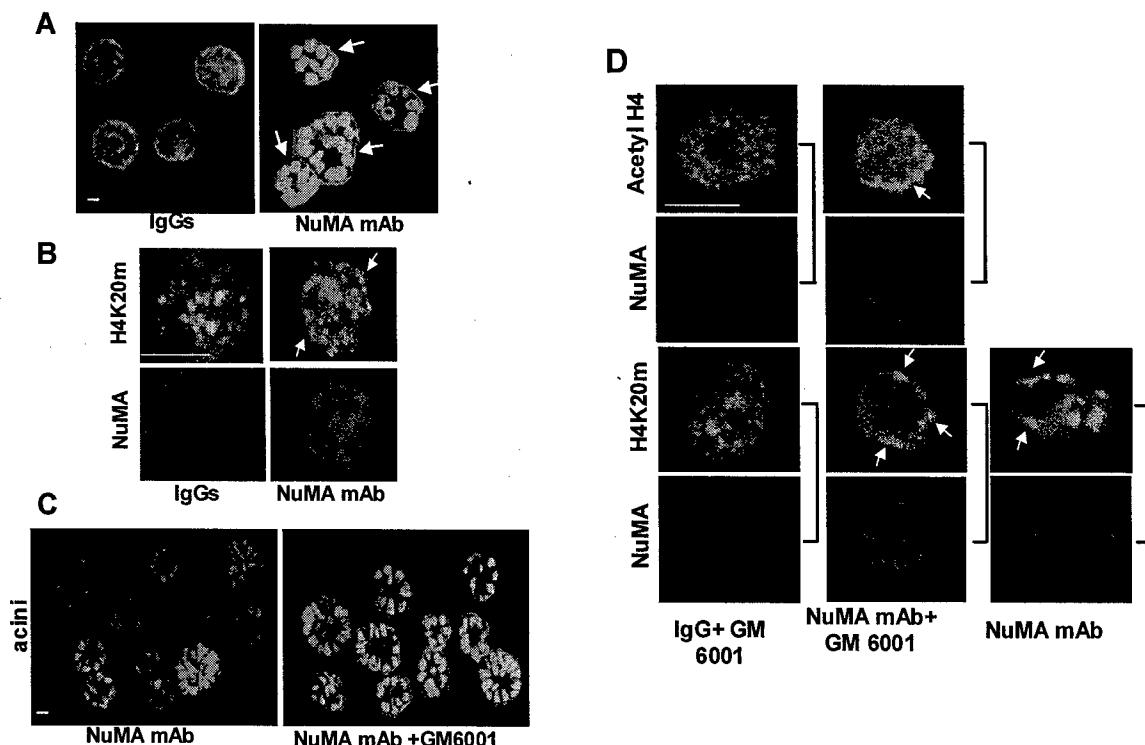


Figure 8. The effect of altering NuMA function on higher order chromatin structure is independent of the loss of basement membrane integrity.

A-D, S1 cells were cultured for 10 days in 3D to induce acinar morphogenesis and incubated with an antibody against NuMA-CT [NuMA mAb] or immunoglobulins [IgGs] for 30 minutes (A-B) or three days (C-D) in the presence or absence of metalloproteinase inhibitor GM6001. Acini were immunostained with an antibody against collagen IV (A and C) to estimate the percentage of acini with basement membrane containing intact collagen IV (green) following incubation with an antibody against NuMA. Since function blocking NuMA antibody, mock IgGs and the antibody against collagen IV are all mouse immunoglobulins, images show staining (green) for collagen IV around the acini (see arrows) and also either staining (green) for IgGs in the cytoplasm (not distinguishable from collagen IV staining, see IgGs in A) or for nuclear anti-NuMA antibody used for treatment of live acini. Nuclei are counterstained with DAPI. Dual immunostaining with secondary antibodies against mouse IgGs to detect NuMA antibodies that entered the cells (red) and immunostaining for acetyl-H4 (green) or H4K20m (green) is shown in B and D. Arrows indicate domains of acetyl-H4 and H4K20m concentrated at the nuclear periphery. Sidebars indicate images that come from the same nucleus. Size bar= 5 microns.

Task 2 (completed): Search for NuMA binding partners.

Our approach for this task encompassed the analysis of NuMA sequence to gain insight into the possible interactions that this protein may have (A) and different experimental strategies to discover potential ligands of NuMA (B).

A. Sequence analysis of NuMA C-terminus

Part of this work has been published (see Abad et al., 2004, in the appendix section) and the other part will be included in a manuscript in preparation.

We were puzzled by the possibility that NuMA may be involved in chromatin structure. So far no enzymatic activity pertaining to chromatin remodeling have been attributed to NuMA. Our current hypothesis is that this protein may act as a receptor to organize multi-protein complexes involved in chromatin remodeling. To gain a better insight into the function of the distal part of NuMA-CT (referred to as NuMA-CTDP) where the HPC2-like domain is located, we exploited established computational techniques and tools to collate and characterize proteins with NuMA-CTDP like regions.

To date, NuMA-like proteins have been identified in *Homo sapiens* (human), *Mus musculus* (mouse), *Rattus norvegicus* (rat), *Bos taurus* (cow), and *Xenopus laevis* (frog). To identify proteins possessing NuMA-CTDP like regions, we used human NuMA residues 1915-2095, designated hNuMA-CTDP, as the protein query for TBLASTN searches against databases of genomic sequences or Expressed Sequence Tags (ESTs). hNuMA-CTDP includes a region missing in the oncogenic NuMA-RAR fusion protein (Wells et al., 1997), and the region described in task 1 believed to be involved in the control of mammary differentiation. We found related sequences showing statistically significant similarity to hNuMA-CTDP in *Sus scrofa* (pig), *Gallus gallus* (chicken), *Danio rerio* (zebrafish), and *Takifugu rubripes* (puffer fish) (See figure 2 in Abad et al., 2004 in appendix). The putative pig and chicken family members were translated ESTs. For puffer fish, part of *Takifugu rubripes* genomic scaffold 307 contained short peptide matches in the -1 frame. This scaffold sequence and the Web version of GENSCAN (Burge and Karlin, 1997) were used to identify an 865 amino acid open reading frame containing the hNuMA-CTDP like region at its C-terminus. The zebrafish hNuMA-CTDP relative was identified in a similar manner using the February 2004 Ensembl assembly of the genome (http://www.ensembl.org/Danio_rerio).

Given the techniques and tools available currently, we found no convincing evidence for hNuMA-CT family members in three other metazoa with fully sequenced genomes (*Caenorhabditis elegans*, *Drosophila melanogaster*, and *Arabidopsis thaliana*). However, we did find what appeared to be a short match to hNuMA-CTDP in a *Ciona intestinalis* genomic scaffold. *Ciona intestinalis* is considered to be one of the earliest chordates because whilst the larval stage has a notochord, it is lost in the adult stage. These observations suggest a relatively broad phylogenetic distribution for members of the NuMA-CTDP family with convincing evidence for their presence in vertebrates (mammals: human, mouse, rat, pig; amphibians: frog; birds: chicken; and teleost fish: zebrafish and puffer fish), and a possibility that NuMA itself might be restricted to the chordate lineage.

To gain insight into the structure and thus, function of NuMA-CTDP, we sought to predict a possible three-dimensional structure for the NuMA-CTDP family. There were no

matches when hNuMA-CTDP was used as the query for a National Center for Biotechnology Information (NCBI) Conserved Domain Database Search. Therefore, we estimated a hidden Markov model (HMM) from a set of diverse NuMA-CTDP sequences, hNuMA-CTDP plus its frog, chicken and teleost relatives (HsNuMA1, XINuMA, GgEST, and FrORF, as shown in figure 2 of Abad et al., 2004 in appendix) using the Sequence Alignment and Modeling (SAM) System (<http://www.soe.ucsc.edu/research/compbio/sam.html>). The resulting NuMA-CTDP HMM was used to search a database of protein sequences of known three-dimensional structure provided by the Research Collaboratory for Structural Biology (RCSB, <http://www.rcsb.org>). The highest scoring protein of the ~55,000 sequences in the February 2004 release from the RCSB was human beta3-integrin (1m1x_B). The region of beta3-integrin identified here is highly conserved in human beta1-and beta6-integrins. The next highest scoring protein was another membrane protein, oncoprotein receptor tyrosine kinase Her2 (1n8y). Although the scores of 1m1x and 1b8y against the NuMA-CTDP HMM were not statistically significant (E values above 1.0), the results are of biological interest because the regions of similarity correspond to largely beta extracellular domains that regulate cell-cell and cell-extracellular matrix interactions, and are involved in signaling (Borges et al., 2000; Hynes, 2002). These results suggest that the three-dimensional structure of NuMA-CTDP might be primarily beta, and that this region could be a site of intermolecular interaction(s).

Further analysis of NuMA-CTDP, showed that proteins found in SWI/SNF chromatin remodeling complexes shared sequence similarities with NuMA (unpublished data). Unfortunately, there is currently no known domain information for the regions of chromatin-associated proteins aligning with NuMA-CTDP. Our current hypothesis is that the distal C-terminus of NuMA may be an important site of interaction with chromatin, possibly by acting as a receptor/organizer for other chromatin-associated proteins.

B. Identification of potential binding partners of NuMA

We have shown that poly-his tagged HPC2-like NuMA-CT peptide used in *in vitro* affinity binding assays trapped a 65-70 kDa ligand from extracts obtained from non-neoplastic S1 cells but not from malignant T4-2 cells. However, the corresponding band on the silver stained gel was very weak. Since it was difficult to get enough material from the 3-D culture of acini to get a signal strong enough to perform mass spectrometry analysis and this technique brings a high background, we decided to also look for potential partners of NuMA using an approach that exploits the observation of the presence of NuMA in the soluble chromatin compartment. We prepared nuclear extracts in the presence of 0.4 M NaCl and ran these extracts on a sucrose gradient that is classically used for separation of multi-protein complexes involved in chromatin remodeling (Tanese et al., 1997). Following a 40hr centrifugation, ten 1.1 ml fractions of the gradient were TCA precipitated and run by SDS-PAGE. We also ran the nuclear pellet, which should contain non-soluble proteins, remaining after preparation of nuclear extracts. NuMA was found in a number of contiguous fractions of the gradient following centrifugation. In contrast, lamin B was only found in the nuclear pellet. We have blotted the different fractions for known components of multi-protein complexes and found that NuMA co-fractionates with components of SWI/SNF multi-protein chromatin remodeling complexes BAF53 and SNF2h (**Figure 9**).

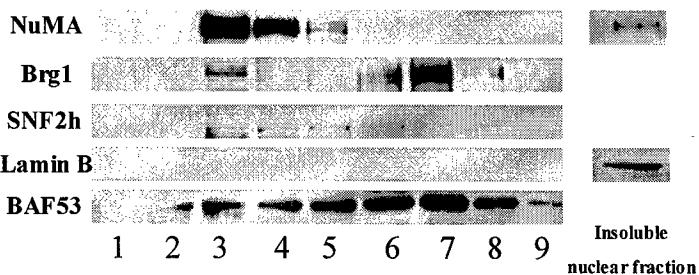


Figure 9. NuMA co-fractionates with BAF53 and SNF2h.
 Nuclear extracts were prepared from S1 acini and centrifuged in a 10-40% gradient sucrose for 40 hours. Fractions 1 to 9 (top to bottom of the gradient) were immunoblotted with antibodies against NuMA, chromatin modifier complex components Brg1, SNF2h, and BAF53, and nuclear matrix protein lamin B. Western blots for NuMA and lamin B with the insoluble nuclear fraction are also shown. Note: bands in lanes 3 and 4 of Brg1 blot correspond to remaining signals from the NuMA blot.

networks (Hanein et al., 1997). The CH domain was originally described at the N-terminus of calponin, an actin-binding protein that regulates smooth muscle contraction (Castresana and Saraste 1995). It is also present as a single copy in signaling proteins, including Vav and IQGAP (Castresana and Saraste 1995; Epp and Chant, 1997). Thus similarly to the SH3 domain, the CH domain represents another case of a protein module present both in cytoskeletal and signaling proteins. We prepared cell extracts to clearly visualize the cytoskeleton in EM and found NuMA present on both actin filaments and intermediate filaments. Based on these preliminary results we believe that the N-terminus of NuMA may serve as an anchor to the cell skeleton, at least when NuMA is in the cytoplasm. Alternatively this region of NuMA could bind to actin or actin-related proteins in the cell nucleus. To the best of our knowledge, so far all NuMA ligands identified with techniques (e.g., the yeast two-hybrid system or co-immunoprecipitation with cell extracts) that cannot test for specific interactions with the cytoskeletal network are only found to bind the C-terminus of NuMA. Therefore instead of searching for potential ligands to NuMA N-terminus in a way similar to that done for NuMA C-terminus (see task 2), we need to specifically investigate NuMA binding to F-actin or cytokeratins; this is beyond the scope of this proposal.

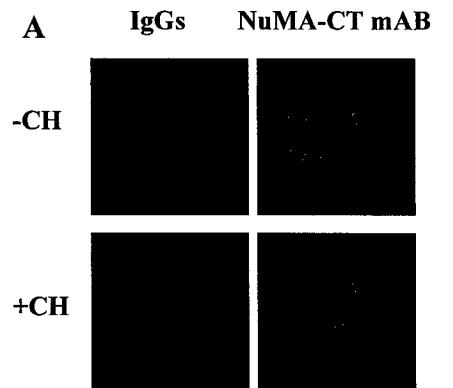
Task 4 (completed): confirmation of the nucleo-cytoplasmic shuttling of NuMA.

Our original experiments using anti-NuMA antibodies to disrupt NuMA organization (Lelièvre et al., 1998), showed that these antibodies directly went to the cell nucleus after gentle digitonin permeabilization of the plasma membrane. Digitonin selectively permeabilizes cellular membranes rich in cholesterol, like the plasma membrane, while intracellular organelle membranes, including the nuclear envelope, with relatively low levels of cholesterol remain intact (Adam et al., 1990; Liu et al., 1999). Then, cells are fixed and stained with a fluorochrome-tagged secondary antibody that permits the visualization (by fluorescence) of the primary antibody incorporated upon digitonin permeabilization. Experiments done with antibodies specifically directed against the N-terminus of NuMA

Task 3 (completed): analysis of NuMA N-terminus for the presence of cytoskeleton binding sequences.

When we started looking at the N-terminal sequence of NuMA in this project, we identified a calponin homology (CH)-binding domain at the very beginning of NuMA sequence. Since then this observation has been published by others (Novatchkova and Eisenhaber, 2002). The CH domain is an actin-binding sequence through which a protein directly interacts with F-actin or related proteins and/or bridges F-actin and intermediate filament

showed that these antibodies first accumulated in the cell nucleus upon digitonin permeabilization of live breast epithelial cells organized into acini, and then returned to the cytoplasm. Since antibodies do not cross the nuclear envelope on their own, this preliminary result led us to hypothesize that the antibodies might be translocating between cytoplasm and nucleus with their antigen, NuMA. As a control we incubated digitonin-permeabilized cells with immunoglobulins (mouse IgGs). Staining with an anti-mouse IgG secondary antibody only showed a diffuse pattern for IgG in the cytoplasm only. We also verified that the 30 second-digitonin treatment did not induce cell death, using trypan blue dye exclusion test and apoptag assay, up to five days after treatment. At the time we were doing these experiments, we also started looking at NuMA distribution with electron microscopy. Surprisingly, electron micrographs revealed the presence of NuMA in both nucleus and cytoplasm. The fact that NuMA is not usually detected in the cytoplasm with low-resolution fluorescence may indicate that it resides in this compartment in small amounts. The presence of NuMA in the cytoplasm was confirmed by soft-X-ray microscopy another high-resolution microscopy. Altogether these results suggested to us that NuMA might be shuttling between nucleus and cytoplasm. To test the hypothesis of NuMA nucleo-cytoplasmic shuttling, we did two types of experiments. First we repeated anti-NuMA antibody translocation experiments in the presence of cyclohexamide, an inhibitor of protein synthesis. The antibody still accumulated in the cell nucleus suggesting that antibody translocation was not associated with de novo synthesis of NuMA (figure 10). We also used monolayer cultures of S1 cells to perform heterokaryon analysis, an assay considered to be the



ultimate evidence for nucleocytoplasmic shuttling (Hache et al., 1999; Fischer et al., 1999). In this assay, human cells

are fused to mouse cells following polyethylene glycol treatment, incubated with cyclohexamide, and fixed at different times for immunostaining (Hache et al., 1999). Immunostaining is performed with an antibody that only recognizes the human isoform of the protein to be tested. Thus, for a protein that usually shows nuclear steady state, if the staining is observed in both the murine nucleus and human nucleus of a fused cell, it indicates that the human isoform of the protein studied has traveled from the human nucleus to the cytoplasm of the heterokaryon and then into the murine nucleus. Murine nuclei are easily recognized by the presence of bright chromatin aggregates as shown by DAPI staining. A few hours following heterokaryon formation between our human S1 cells and NIH-3T3 mouse cells, immunostaining performed with an antibody that only recognizes the human isoform of NuMA (clone 107.7, a kind gift from Dr. Nickerson,

Figure 10. NuMA shuttles in differentiated cells in the presence of an inhibitor of protein translation. Nonmalignant S-1 cells were allowed to differentiate into acini in 3D culture for 10 days. Live acini were incubated without (-CH) or with (+CH) cyclohexamide for three hours to block protein translation, then permeabilized with 0.01% digitonin in permeabilization buffer and incubated with control immunoglobulins (IgGs) or antibodies against the C-terminus of NuMA (NuMA-CT mAb) in the absence (-CH) or presence (+CH) of cyclohexamide for 12 hours. After 12 hours, immunostaining with Texas Red-conjugated secondary antibody showed that the antibody had reached the nucleus in both the absence and presence of cyclohexamide, while IgGs remain cytoplasmic.

Umass) showed the presence of NuMA in both murine and human nuclei (**Figure 11**), thus confirming NuMA nucleo-cytoplasmic shuttling.

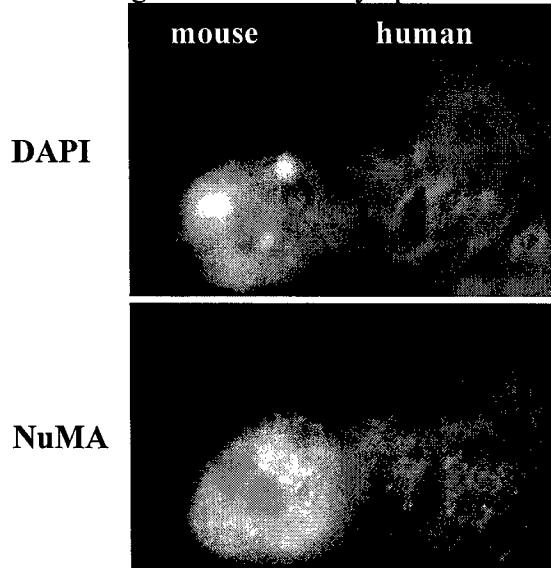


Figure 11. Heterokaryon analysis shows that NuMA shuttles between nucleus and cytoplasm in breast epithelial cells cultured as a monolayer (2D culture). Nonneoplastic S1 cells and NIH-3T3 murine cells were co-cultured for 2 days, fused using polyethylene glycol and treated with cyclohexamide to prevent de novo protein synthesis. Cells were fixed and immunostained at different time intervals with an antibody that recognizes only the human isoform of NuMA (clone 107.7). A murine nucleus is identified by its bright speckles next to a human nucleus (DAPI, upper image). NuMA staining is found in both the murine and human nuclei (NuMA lower image). NuMA staining could not be detected in isolated murine nuclei (control with non-fused cells, not shown).

TIMETABLE

Task 1: Completed

Task 2: completed

Task 3: completed

Task 4: completed

KEY RESEARCH ACCOMPLISHMENTS

- The distal portion of NuMA-CT (NuMA CTDP) that encompasses the HPC2-like domain seems restricted to vertebrates and is predicted to have a primarily beta strand three-dimensional structure that favors interaction with other proteins. Sequence similarities with several chromatin-binding proteins are found within NuMA-CTDP.
- NuMA shuttles between nucleus and cytoplasm
- Non-neoplastic S1 cells transfected with NuMA-CTDP do not differentiate properly (lack of complete basement membrane) and show altered chromatin organization. In contrast, insertless vector and NuMA-CTDP without the NLS do not affect chromatin structure or acinar differentiation.
- The relationship between NuMA and chromatin is altered in cells that do not differentiate into acini.

- NuMA-CTDP sequence was expressed in bacteria and purified as a native poly-his fusion peptide.
- Affinity binding assay with poly-his NuMA-CTDP fusion peptide indicates the presence of a ligand for this sequence in nonmalignant cells; this ligand is not found in the malignant counterpart.
- NuMA is found in the soluble chromatin compartment.
- NuMA is found in fractions of nuclear extracts containing multi-protein chromatin remodeling complexes of the SWI/SNF family.

REPORTABLE OUTCOMES

- 1) Development of cell lines: nonmalignant S1 cells expressing NuMA-CTDP
- 2) Production of purified poly-His NuMA-CTDP fusion peptide
- 3) A US patent (# 6,287,790 B1) based on the work previously supported by DOD (to Sophie A. Lelièvre) was issued in September 2001. The patent deals mostly with the use of NuMA distribution as a marker of cancer progression.
- 4) The P.I., Sophie Lelièvre, was recruited as a Walther Assistant Professor in the Department of Basic Medical Sciences, School of Veterinary Medicine at Purdue University (West Lafayette, IN). The starting date was October, 2, 2000. The appointment is a joint appointment between the Department of Basic Medical Sciences and the Walther Cancer Institute for the first three years (exceptionally for four years), then the appointment in 100% in the Department of Basic Medical Sciences.
- 5) Sophie Lelièvre presented some of the NuMA work as part of her research program presentation in front of an external review panel gathered to evaluate the status of Walther Cancer Institute (WCI) investigators. Her status was maintained and later on exceptionally renewed for a 4th year due to good performance. (WCI 25% salary support for young investigators is usually set up for three years).
- 6) Patricia Abad the research assistant working on the project since the beginning of 2001, was promoted to research professional (AP) level in the Fall of 2001. She was also accepted into graduate school and worked has a part time Master's student and full time AP (until August 2003) on the NuMA project.
- 7) Patricia Abad, the research assistant working on the NuMA project obtained her Master's degree in August 2003. She was then accepted in the PhD program and continued to work on the NuMA project in the Lelièvre laboratory after obtaining a Purdue Doctoral Fellowship.
- 8) For her work on NuMA, Patricia Abad received an Honorable Mention at a university wide Sigma Xi poster presentation in 2002.
- 9) In 2003, Patricia Abad received an Award from the Walther Cancer Institute for her presentation on the role of NuMA in the control of mammary phenotype and chromatin organization during a regional competition involving oral presentation from graduate students.
- 10) In 2004, Patricia Abad received a travel Award from the American Society for Cell Biology (ASCB), to present a poster of her work on NuMA at the ASCB annual meeting.

- 11) Patricia Abad has been invited to present a poster of her work on NuMA at the Era of Hope meeting in June 2005.
- 12) Two undergraduate students, Zoltan Metlagel and Aniysha Nelpurackal, obtained their Bachelor's degree with honors due to their work on the NuMA project. They are now enrolled in graduate programs at other universities. Zoltan worked on the bacterial expression of poly-His NuMA-CTDP fusion protein and affinity binding assay with financial support from a summer fellowship (2002) from the Carroll County Cancer Association. Aniysha worked on the cell membrane fractionation experiments presented in the Abad et al., 2004 publication, with support from a Howard Hughes fellowship for undergraduate research (2003).
- 13) Two other undergraduate students, Jason Lewis and Lesley Chaboub also worked on the NuMA project for research credits. In addition, Jason was a recipient of the DOD undergraduate training fellowship at Purdue for the summer of 2003. Jason is now applying to Medical School and Lesley is in Graduate School.
- 14) Based on the novel data suggesting a role for NuMA in chromatin structure, an RO1 application was submitted to NIH in February 2004 to explore the mechanism of NuMA function at the chromatin level and alterations of such functions upon tumor development. This first RO1 application received a score and percentile, and it will be resubmitted this summer.
- 15) The presence of NuMA in the chromatin compartment may explain the wide range of NuMA distributions observed with different cell phenotypes. We exploited this observation to design a novel image analysis of NuMA distribution in collaboration with David Knowles at the Lawrence Berkeley National Laboratory. The image analysis method now enables us to characterize NuMA distribution by a simple graphic representation and 'recognize' cell phenotypes based on NuMA distribution. This automated analysis will be extended to other nuclear proteins and tested for the classification of breast neoplasias on biopsies. Three different projects have been funded (one to Dr. Knowles and two to Dr. Lelièvre) on that aspect. A manuscript on this work has been submitted for publication [Knowles, D Sudar, CM Bator Kelly, MJ Bissell and SA Lelièvre. "Novel image analysis links the sub-nuclear distribution of NuMA with alterations in mammary cell phenotype".].
- 16) A small grant was obtained from the Purdue Research Foundation to pursue the novel direction of research on NuMA shuttling derived from results obtained in task 4.
- 17) There are five manuscripts in which support for this project is acknowledged:
 - (a) A review discussing novel directions in breast cancer research partly based on the data obtained in this project was published in June 2003 [C Plachot and SA Lelièvre. "New directions in tumour biology: from basement membrane-directed polarity to DNA Methylation". Mathematical Biology and Medicine Series, "Cancer modeling and simulation", Chapman & Hall/CRC, 2003.]
 - (b) A manuscript on the analysis of NuMA-CT sequence was published in 2004 [PC Abad, IS Mian, C Plachot, A Nelpurackal, C Bator-Kelly, and SA Lelièvre. "The C-terminus of the nuclear protein NuMA: phylogenetic distribution and structure". *Protein Sci.* 13: 2573-2577, 2004]

(c) A peer-reviewed chapter that discusses the development and use of three-dimensional cell culture, and contains significant information gathered thanks to the NuMA project, was written in collaboration with Dr. Mina Bissell. This manuscript has been accepted for publication [SA Lelièvre and MJ. Bissell. Three dimensional cell culture: The importance of context in regulation of function. *Encyclopedia of Molecular Cell Biology and Molecular Medicine (EMCBMM)*. (In press, 2005)].

(d) A manuscript that deals with the function of NuMA-CT in mammary acinar differentiation and chromatin structure is about to be submitted [PC Abad, J Lewis, IS Mian, S Badve, J Xie, and SA Lelièvre. "The nuclear apparatus protein NuMA impacts higher order chromatin structure in mammary epithelial morphogenesis"].

(e) Another manuscript that explores the relationship between higher order nuclear structure and tissue polarity and includes some of the findings from the work developed in the NuMA project will be submitted shortly [G Chandramouly, PC Abad, and SA Lelièvre. "Restoration of higher order nuclear structure characteristic of phenotypically normal differentiation is critical for the reversion of tumor phenotype."].

18) Abstracts (13 abstracts related to the NuMA project were presented at local, national and international meetings).

1. NuMA shuttles in breast differentiated tissue. **Patricia Abad**, Annie Viron, Jeffrey Nickerson, Edmond Puvion, Mina J. Bissell, and **Sophie Lelièvre**. American Society for Cell Biology meeting 2001 8-12 December, Washington D.C.
2. The subcellular compartmentalization of the nuclear mitotic apparatus protein NuMA regulates differentiated and tumor behaviors. **Patricia Abad**, V.M. Weaver, M.J. Bissell, and **Sophie Lelièvre**. 4th Annual Amelia Project Retreat (Catherine Peache Fund) February 2nd, 2002, Indianapolis, IN.
3. NuMA shuttles between nucleus and cytoplasm in breast differentiated tissue. **Patricia Abad**, Annie Viron, Jeffrey Nickerson, Edmond Puvion, Mina J. Bissell, and **Sophie Lelièvre**. Phi Zeta Day, April 2, 2002, Purdue University, West Lafayette IN.
4. Nucleo-cytoplasmic shuttling of nuclear apparatus protein NuMA in breast differentiated tissue. **Patricia Abad**, Annie Viron, Jeffrey Nickerson, Edmond Puvion, Mina J. Bissell, and **Sophie Lelièvre**. Sigma Xi Day, February 26, 2002, Purdue University, West Lafayette IN.
5. Protein compartmentalization in phenotypically normal and malignant breast epithelial cells: A source for the identification of novel targets for anti-cancer strategies. **Patricia C. Abad**, Saira Mian, Mina J. Bissell, and **Sophie A. Lelièvre**. ERA of Hope, Department of Defense/BCRP Meeting, September 2002, Orlando, FL.

6. Implications of NuMA shuttling for Human mammary epithelial cell differentiation and breast cancer. **Patricia Abad**, Jeffrey A. Nickerson, and **Sophie Lelièvre**. Walther Cancer Institute Annual Scientific Retreat August 8-10, 2002, Purdue University, West Lafayette, IN.
7. Identifying NuMA binding partners involved in mediating mammary epithelial cell survival. **Patricia Abad**, Zoltan Metlagel, Valerie Weaver, Saira Mian, Ali Ravanpay, Mina Bissell, and **Sophie Lelièvre**. 42nd annual American Society for Cell Biology Meeting December 14– 18 2002, San Francisco, CA.
8. Identifying NuMA ligands engaged in mammary epithelial cell differentiation. **Patricia Abad**, Zoltan Metlagel, Valerie Weaver, Saira Mian, Ali Ravanpay, Mina Bissell, and **Sophie Lelièvre**. 5th Annual Amelia Project Retreat (Catherine Peachey Fund) February 1, 2003, Indianapolis, IN.
9. The C-terminal sequence in the nuclear protein NuMA acts as a functional link between integrin adhesion and nuclear structure to regulate survival in breast acini. **Patricia Abad**, Valerie Weaver, Saira Mian, Mina Bissell, and **Sophie Lelièvre**. 2003 Walther Cancer Institute Annual Retreat August 7-9, 2003, Indianapolis, IN.
10. Implications of NuMA carboxi-terminus in breast acinar differentiation and cancer. **Patricia Abad**, Jason Lewis, Saira Mian, and **Sophie Lelièvre**. 6th Amelia Research day, Indianapolis, February 7, 2004.
11. Phenotypical reversion of breast tumor cells is accompanied by the restoration of higher order nuclear organization characteristic of phenotypically normal non-neoplastic cells. **Gurushankar Chandramouly**, **Patricia Abad**, and **Sophie Lelièvre**. 44th Annual American Society for Cell Biology Meeting December 4-8, 2004.
12. A Novel Role for the Nuclear Apparatus Protein NuMA in Higher Order Chromatin Organization in Breast Epithelium. **Patricia Abad**, Jason Lewis, Saira Mian, and **Sophie Lelièvre**. 44th Annual American Society for Cell Biology Meeting December 4-8, 2004.
13. Role of Higher Order Nuclear Structure in Breast Tumor Cell Reversion. **Gurushankar Chandramouly**, **Patricia Abad**, and **Sophie Lelièvre**. 7th Annual Amelia Project Retreat (Catherine Peachey Fund) February 5, 2005, Indianapolis, IN.
- 19) Oral presentations given by the P.I. and related the project: nine invited seminars/talks at national and international institutions or meetings.

Presentations at meetings:

“NuMA functionally links cell adhesion and nuclear structure to regulate cell survival in breast”, 2nd International Conference on Tumor Microenvironment: Progression, Therapy and Prevention, (Baden, Austria), June 2002;

“Multiple facets of nuclear structural proteins: The Role of NuMA in the regulation of breast epithelial phenotypes”, 12th International Conference of the International Society of Differentiation, (Lyon, France), September 2002;

“Link between compartmentalization and functions of nuclear proteins in phenotypically normal and neoplastic tissues”, FASEB Summer Research Conference on “Nuclear Structure and Cancer”, (Saxtons River, VT), June 2003;

“Tissue structure and gene expression control”, Research in Cell Therapy Workshop, Session on proliferation and differentiation in normal and pathological cells, (St Louis Hospital, Paris, France), March 2004

Seminars:

“Structure, Instability and Plasticity in Cancer” University of Mexico, (Mexico City), September 2001;

“Subcompartmentalization of Nuclear Proteins in Differentiation and Cancer: Multi-faceted NuMA Regulates Breast Epithelial Cell Behavior.” IUPUI, (Indianapolis, IN), November 2001;

“Chromatin structure and breast differentiation: The role of the supramolecular organization of nuclear proteins” Boston University, (Boston, MA), April 2003;

“Architectural proteomics –When proteins become stars”, Lawrence Berkeley National Laboratory, Life Sciences Division, (Berkeley, CA), February 2004;

“Three-dimensional culture of non-neoplastic and neoplastic breast tissues to unravel higher order control of proliferation, survival and differentiation”, Georgetown University, School of Medicine (Washington, DC), January 2005

PERSONNEL PAID VIA THE AWARD

Part of Dr. Lelièvre's salary

80 % of Patricia Abad's AP salary until Summer 2003.

CONCLUSIONS

In this research work, we have demonstrated that NuMA was part of the chromatin compartment and was involved in the control of chromatin structure. The distal portion of the C-terminus of NuMA (NuMA-CTDP) seems to be critical for the relationship between NuMA and chromatin. The presence of NuMA-CTDP family members in vertebrates, and possibly the early chordate *Ciona intestinalis*, together with their likely absence in invertebrates, suggests that NuMA-CTDP may have a vertebrate-specific role. Given that NuMA has a role in differentiation (Lelièvre et al., 1998; Sukhai et al., 2004), one hypothesis

is that NuMA-CTDP may be associated with the control of gene expression. This hypothesis is supported by the fact that expression of NuMA truncated at its C-terminus and antibodies directed against NuMA-CT induce alterations in chromatin organization (Gueth-Hallonet et al., 1998; Lelièvre et al., 1998), and by our current data showing that NuMA is part of the chromatin compartment and that NuMA-CT may be involved in the interaction between NuMA and the chromatin compartment. We have also unraveled shuttling properties in NuMA (task 4). These particular data have led to the development of a new research project (funded by a small grant from the Purdue Research Foundation) that aims at analyzing the function of NuMA shuttling.

Altogether, data presented in tasks 1 and 2 indicate a critical role for NuMA in chromatin organization and the control of mammary epithelial differentiation. These data are the basis of an NIH R01 proposal that was submitted with the aim of identifying the molecular mechanisms by which NuMA influences chromatin structure and how NuMA function may be altered upon breast cancer development. Importantly, our work brings a possible rationale for how alterations in NuMA, that have been recently linked to increased breast cancer risk (Kammerer et al., 2005), may be involved in breast cancer development. Further investigations on NuMA function are based on the fact that proteins bearing actin-binding domains have been recently proposed to play a critical role in the control of gene expression by providing a structural framework that facilitates and integrates molecular cross-talk within the nucleus (Shumaker et al., 2003). Interestingly, it was reported that NuMA possesses a calponin homology (CH) domain at its N-terminus (Novatchkova and Eisenhaber, 2002). The particular structure of NuMA containing actin-binding (CH domain), structural (central coiled-coil domain) and organizational and signaling potential (NuMA-CTDP region) suggests that NuMA may provide a structural platform for coordinating processes in the nucleus and possibly transducing cytoplasmic signals. Thus, NuMA may become an important target in future investigations focusing on the development of differentiation strategies in breast cancer.

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Appendix

- (1) Sophie Lelièvre's Curriculum Vitae
- (2) pdf format of Abad et al., 2004
- (3) pdf format of Plachot and Lelièvre, 2003

CURRICULUM VITAE

NAME	POSITION TITLE
Sophie A. Lelièvre	Walther Assistant Professor of Basic Medical Sciences

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE (if applicable)	YEAR(s)	FIELD OF STUDY
University of Louvain, Belgium	Engineer	1984-1987	Veterinary Sciences
University of Liège, Belgium	Medical Degree	1987-1990	Veterinary Medicine
University of Paris VI, France	Master's	1990-1991	Molecular and Cell Pharmacology
University of Paris VI, France	Ph.D.	1991-1994	Molecular and Cell Pharmacology
Lawrence Berkeley Natl Lab, Berkeley, USA	postdoc	1995-1999	Mammary Cell Biology

RESEARCH AND PROFESSIONAL EXPERIENCE

1988-1990: Trainee	School of Veterinary Medicine (Cureghem, Bruxelles, Belgium) Topics: Canine mammary cancer pathology and chemotherapy
1991-1995: Veterinary Surgeon	Pets Emergency Room, Paris District, France
1991-1994: Predoctoral Fellow	Gustave Roussy Cancer Institute (Villejuif, France) Topics: Topoisomerases, anti-cancer pharmacology
1991-1994: Teaching assistant	University of Paris; topics: Embryology and Histology
1995: Postdoctoral Fellow	Gustave Roussy Cancer Institute (Villejuif, France), and a 3-month training in Dr. Kohwi-Shigematsu's laboratory (LJCRF, La Jolla, CA). Topics: Resistance to topoisomerase inhibitors, metastatic phenotype, M.A.R., nuclear matrix
1995-1997: Postdoctoral Fellow	Lawrence Berkeley National Laboratory; Dr. Mina Bissell's laboratory (Berkeley, CA)
1997-1999: Postdoctoral Scientist	Topics: Extracellular matrix-nuclear structure interrelationship, regulation of gene expression in breast morphogenesis and tumorigenesis
1999-2000: Research Scientist	Cell and Molecular Biology Dept., Lawrence Berkeley National Lab. (Berkeley, CA) Topics: Nuclear organization and gene expression, nuclear signaling
10/2000-ongoing: Assistant Professor	Basic Medical Sciences, Purdue University, West Lafayette, IN Topics: Nuclear organization in breast differentiation and cancer, nuclear signaling, nuclear structure and genomic instability

2003-ongoing: **IU Medical School adjunct** Cancer Pharmacology, Indiana University School of Medicine, Assistant Professor Indianapolis, IN

HONORS

University of Louvain (Belgium), lifetime tuition exemption for outstanding student, 1985

National Prize for Fundamental Cancer Research/young investigator, 1995 (French Society of Cancer and National Federation of Cancer Institutes, France)- *One Prize awarded annually; paper-based competition in which trainees present ongoing and future research goals- It was awarded to me because of data showing for the first time the importance of three-dimensional organization of cells in the development of tumors showing levels of resistance comparable to clinical situations and in the acquisition of increased metastatic potential, and also for the demonstration that resistance to anti-cancer drug topoisomerase II inhibitors was accompanied by changes in the nuclear compartmentalization of topoisomerase II beta. The future goals emphasized the importance of studying the role of changes in nuclear structure and in the compartmentalization of nuclear proteins in cancer cell behavior.*

National Alexandre Joel Prize for young investigator, 1995 (Association for Cancer Research, ARC, France)- *One Prize awarded annually only if a suitable postgraduate candidate is identified. Award is made upon nomination by established scientists and reference letters that describe the accomplishments of the young investigator in the domain of cancer research.*

Lawrence Berkeley National Laboratory Outstanding Performance Award, 1998 (Lawrence Berkeley National Laboratory, Berkeley, CA)- *Award received for significant contribution to the development of the use of soft X-ray microscopy to study cellular (including nuclear) structure.*

Integrated Science Partnership Program Appreciation Award, 1999 (Lawrence Berkeley National Laboratory, Berkeley, CA)

Walther Support for New Investigator, 2000-2004 (Walther Cancer Institute, Indianapolis, IN)- *Selected candidates who are recruited as assistant professors at Purdue University receive support from Walther Cancer Institute for three years, extendable to a fourth year upon satisfactory performance. Support was received for four years.*

Teaching Fellowship: "Moniteur" position, University of Paris, 1991-1994- *Fellowship only awarded to top-ranked students (ranked upon completion of their Master's), accepted in a Ph.D. program and already selected for a graduate fellowship from the French Ministry of Education and Research. This program includes regular teaching assignments and workshops on teaching methods, pedagogy, and history of Science.*

Research Fellowships:

French Ministry of Education and Research (France), graduate fellowship, 1991-1994

International Agency for Research on Cancer (IARC-WHO), postdoctoral fellowship, 1995-1996

Association for Cancer Research (ARC), complementary fellowship, 1996

Department Of Defense/USA-Breast Cancer Research Program, postdoctoral training grant, 1997-1999

Collaborative Research Fellowships and Travel Awards:

French Society of Cancer Travel Fellowship, 1995

Journal of Cell Science Travel Fellowship, 1997

Philippe Foundation Travel Fellowship, 1998

Purdue University International Travel Award, 2001 and 2002

International Society of Differentiation Travel Award, 2002***Session Chair at scientific meetings:***

Session on "Cellular Organization, Signal Transduction and Cancer" at the "Biology and Mathematics of Cells: Physiology, Kinetics and Evolution" European Society for Mathematical and Theoretical Biology (ESMTB) meeting, Spain, 2001

Co-organizer and session co-chair on "Nuclear Compartmentalization in Differentiation and Cancer" at the International Society of Differentiation meeting, France, 2002

Panelist and reviewer for funding agencies

Invited panelist on Focused Discussion Group – Breast Cancer, Walther Cancer Institute, 2003

Ad hoc reviewer, 2004-on: Department of Defense/Breast Cancer Research Program and Prostate Cancer Research Program; Komen Foundation for Breast Cancer Research; Department of Health (Florida State)

INVITED PRESENTATIONS***Speaker at regional, national, and international meetings:***

"The solid-state signaling pathway from the extracellular matrix to the nuclear matrix: the critical role of 3D architecture at the cellular level", High resolution X-ray CMT Workshop (LBNL, Berkeley, CA), August 1996;

"Internal cell architecture-A new look", Advanced Light Source Users Meeting (LBNL, Berkeley, CA), October 1997;

"Global rearrangement of nuclear matrix-associated proteins when human mammary epithelial cells are cultured in 3-D: an analysis using confocal-, electron-, and soft x-ray microscopy", Keystone Symposium on Nuclear Matrix (Copper Mountain, CO; junior investigators workshop), April 1998;

"Nuclear structure, cell proliferation, and tissue morphogenesis", American Society for Cell Biology Meeting (San Francisco, CA), December 1998;

"Tissue architecture and gene expression: study of tissue matrix in three-dimensional models of cell culture" and ***"The non-chromatin structure of the nucleus or nuclear matrix: study of its interaction with the chromatin structure and its role in the regulation of gene expression"***, Biomathematics Summer School (Termoli, Italy), Mathematics in Cell Physiology and Proliferation, June 1999;

"Nuclear-directed signaling in mammary gland acini", Gordon Conference on Biological Structure and Gene Expression (Meriden, NH; short talk), August 1999;

"Nuclear organization in normal and malignant breast: NuMA is a marker of cell phenotype and a regulator of differentiation", Era of Hope DOD Breast Cancer Research Meeting (Atlanta, GA; platform talk), June 2000;

"Cell cycle regulation in higher order cell assemblies: the role of three-dimensional tissue architecture," Third International Congress of Nonlinear Analysts (Catania, Sicily), July 2000;

"Signal transduction and feedback signaling", ***"Cellular transformation and genomic instability"***, and ***"Tumor progression: How in vitro models may help understand in vivo situations"***, ESMTB School, Biology and Mathematics of Cells: Physiology, Kinetics and Evolution, (Siguenza, Spain), June 2001;

"NuMA functionally links cell adhesion and nuclear structure to regulate cell survival in breast", 2nd International Conference on Tumor Microenvironment: Progression, Therapy and Prevention, (Baden, Austria), June 2002;

"Multiple facets of nuclear structural proteins: The Role of NuMA in the regulation of breast epithelial phenotypes", 12th International Conference of the International Society of Differentiation, (Lyon, France), September 2002;

"Link between compartmentalization and functions of nuclear proteins in phenotypically normal and neoplastic tissues", FASEB Summer Research Conference on "Nuclear Structure and Cancer", (Saxtons River, VT), June 2003;

"Proteomics with a twist-Early detection of breast cancer by looking beyond protein expression", The Amelia Project-Giving Wings to Research, (Indianapolis, IN), February 2004;

"Tissue structure and gene expression control", Research in Cell Therapy Workshop, Session on proliferation and differentiation in normal and pathological cells, (St Louis Hospital, Paris, France), March 2004

Seminars:

"The nuclear matrix is an old concept still in its infancy", Gustave Roussy Cancer Institute, Dept. of Clinical and Molecular Pharmacology, (Villejuif, France), September 1995;

"The solid-state pathway: a model for the regulation of gene expression", University of Paris XII, CRRET Laboratory, (Créteil, France), June 1996;

"From the extracellular matrix to the nuclear matrix, the dynamic cellular architecture plays a role in the regulation of cellular behavior: a study of a model of mammary tumorigenesis" Gustave Roussy Cancer Institute, Dept. of Clinical and Molecular Pharmacology, (Villejuif, France), June 1996;

"Dynamic re-organization of nuclear architecture during tumorigenesis and tumor reversion", Harvard Children's Hospital, (Boston, MA), April 1997;

"The role of cellular and tissue structure during tumorigenesis", Institute of Immunology, (Munich, Germany), June 1997;

"Dynamic reciprocity between the extracellular matrix and the organization of the cell nucleus: a study of mammary epithelial cell morphogenesis", Institute Molecular Genetics, (Paris, France), June 1998;

"Interrelationships between the distribution of nuclear matrix proteins, chromatin structure and gene expression during mammary epithelial cells morphogenesis", Center for Atomic Energy (CEA), (Fontenay aux Roses, France), June 1998;

"Communication between the extracellular matrix and the nuclear structure in breast development and malignancy", Boston University Medical School, (Boston, MA), Dept of Biochemistry, February 1999;

"The role of nuclear organization in normal and malignant breast structures", California Pacific Medical Center Research Institute (San Francisco, CA), May 1999;

"Nuclear organization in normal and malignant breast", Division of Radiation and Cancer Biology, New England Medical Center, TUFTS University, (Boston, MA), 1999;

"What is the link between nuclear architecture and the expression of malignancy?" Purdue University, Dept. of Basic Medical Sciences (West Lafayette, IN), March 2000;

"The organization of the cell nucleus in breast differentiation and tumorigenesis. A source for the development of novel anticancer strategies," Research Institute of Molecular Pathology, Vienna Biocenter, Boehringer-Ingelheim, (Vienna, Austria), April 2000;

"Structure, Instability and Plasticity in Cancer" University of Mexico, (Mexico City), September 2001;

"Subcompartmentalization of Nuclear Proteins in Differentiation and Cancer: Multi-faceted NuMA Regulates Breast Epithelial Cell Behavior." IUPUI, (Indianapolis, IN), November 2001;

"Chromatin structure and breast differentiation: The role of the supramolecular organization of nuclear proteins" Boston University, (Boston, MA), April 2003;

"Architectural proteomics -When proteins become stars", Lawrence Berkeley National Laboratory, Life Sciences Division, (Berkeley, CA), February 2004;

"Three-dimensional culture of non-neoplastic and neoplastic breast tissues to unravel higher order control of proliferation, survival and differentiation", Georgetown University, School of Medicine (Washington, DC), January 2005

PATENTS

SA Lelièvre and MJ Bissell. "Utilization of nuclear structural proteins for targeted therapy and detection of proliferative and differentiation disorders". US 6,287,790 B1, Sep.11, 2001.

PUBLICATIONSFrom Graduate Work

K Bojanowski, **S Lelièvre**, J Markovits, J Couprie, A Jacquemin-Sablon and AK Larsen, "Suramin is an inhibitor of DNA topoisomerase II *in vitro* and in Chinese hamster fibrosarcoma cells". *Proc.Natl.Acad.Sci., USA*, 89:3025-3029, 1992.

S Lelièvre and AK Larsen, "Development and characterization of suramin-resistant Chinese hamster fibrosarcoma cells: drug-dependent formation of multicellular spheroids and a greatly enhanced metastatic potential." *Cancer Res.*, 54: 3993-3997, 1994.

S Lelièvre and AK Larsen, "Suramin resistance in Chinese hamster fibrosarcoma cells is accompanied with morphological alterations and metastases formation." *Bull. Cancer*, 81: 903-905, 1994.

S Lelièvre and AK Larsen, "Chronic *in vitro* suramin exposure leads to the development of drug resistant sublines which grow as three dimensional cultures and are highly invasive *in vivo*. Lack of growth factor involvement in the cytotoxic action of the drug." In "Novel approaches in anticancer drug design. Molecular modelling-New treatment strategies. *Contrib. Oncol.*, 49: 117-123, 1995, (WJ Zeller, D'Incàlci M, and Newell DR, eds), Basel, Karger.

S Lelièvre, Y Benchokroun, and AK Larsen, "Altered DNA topoisomerase I and II in suramin-resistant Chinese hamster fibrosarcoma cells." *Mol. Pharmacol.*, 47: 898-906, 1995.

From Postdoctoral Work

***S Lelièvre**, VM Weaver, and MJ Bissell, "Extracellular matrix signaling from the cellular membrane skeleton to the nuclear skeleton: a model of gene regulation in mammary epithelial cells." *Recent Progress in Hormone Research*, 51:417-432, 1996.

***S Lelièvre** and MJ. Bissell. "The solid-state signaling pathway from extracellular matrix to nuclear matrix: the critical role of three-dimensional architecture for functional differentiation." Proceedings of the 1996 Workshop on High Resolution Computed Tomography (CMT), LBNL/UC, pp 85-96, 1997.

***S Lelièvre**, VM Weaver, CA Larabell, and MJ Bissell, "Extracellular matrix and nuclear matrix interactions may regulate apoptosis and tissue-specific gene expression: a concept whose time has come." In *Advances in Molecular and Cell Biology: Cell Structure and Signaling*, (RH Getzenberg, ed), JAI Press Inc, Greenwich CT, Vol 24, pp: 1-55, 1997.

SA Lelièvre, VM Weaver, JA Nickerson, CA Larabell, A Bhaumik, OW Petersen, and MJ Bissell. "Tissue phenotype depends on reciprocal interactions between extracellular matrix and the structural organization of the nucleus" *Proc. Natl. Acad. Sci. (USA)*, 95: 14711-14716, 1998.

***SA Lelièvre** and MJ Bissell. "Communication between the cell membrane and the nucleus: the role of

protein compartmentalization" 25th Anniversary Issue of *J. Cell. Biochem*, 30/31 suppl.: 250-263, 1998.

*MJ Bissell, VM Weaver, **SA Lelièvre**, F Wang, OW Petersen, and KL Schmeichel, "Tissue structure, nuclear organization and gene expression in normal and malignant breast" *Cancer Res.*(SUPPL), 59: 1757s-1764s, 1999.

H-M Chen, KL Schmeichel, IS. Mian, **SA Lelièvre**, OW Petersen, and MJ Bissell. AZU-1: a candidate breast tumor suppressor and biomarker for tumorigenic reversion. *Mol.Biol.Cell*, 11: 1357-1367, 2000.

SA Lelièvre, MJ Bissell, and P Pujuguet. "Cell nucleus in context." *Crit. Rev. Eukar. Gene Expression*, 10: 13-20, 2000.

W Meyer-Ilse, D Hamamoto, A Nair, **SA. Lelièvre**, G Denbeaux, L Johnson, A Lucero, D Yager, and CA. Larabell. "High Resolution Protein Localization Using Soft X-ray Microscopy." *J. Microscopy*, 201: 395-403, 2001.

C Ortiz de Solorzano, R. Malladi, **SA Lelièvre**, and SJ Lockett. "Segmentation of nuclei and cells using membrane related protein markers." *J. Microscopy*, 201: 404-15, 2001.

SK Muthuswamy, D Li, **SA Lelièvre**, MJ Bissell, and J Brugge. "ErbB2, but not ErbB1, can reinitiate proliferation and induce luminal repopulation in growth-arrested epithelial acini." *Nature Cell Biology*, 3: 785-792, 2001.

VM Weaver, **SA Lelièvre**, JN Lakins, MA Chrenk, J Jones, F Giancotti, Z. Werb, and MJ Bissell. "Beta4-integrin-dependent formation of polarized three-dimensional architecture confers resistance to apoptosis in normal and malignant mammary epithelium". *Cancer Cell*, 2: 205-19, 2002.

Since independent position (October 2000)

C Plachot and **SA Lelièvre**. "New directions in Tumour biology: from basement membrane-directed polarity to DNA Methylation". In Mathematical Biology and Medicine Series, "Cancer modeling and simulation", Chapman & Hall/CRC, 2003.

C Plachot and **SA Lelièvre**. "DNA methylation control of tissue polarity and cellular differentiation in the mammary epithelium" *Exp. Cell Res.*, 298: 122-32, 2004.

PC Abad, IS Mian, C Plachot, A Nelpurackal, C Bator-Kelly, and **SA Lelièvre**. "The C-terminus of the nuclear protein NuMA: phylogenetic distribution and structure". *Protein Sci.* 13: 2573-2577, 2004.

P Kaminker, C Plachot, S-H Kim, P Chung, D Crippen, OW Petersen, MJ Bissell, J Campisi and **SA Lelièvre**. "Higher order nuclear organization in growth arrest of human mammary epithelial cells: A novel role for telomere-associated protein TIN2". *J. Cell Sci.* 118: 1321-1330, 2005.

SA Lelièvre and MJ. Bissell. Three dimensional cell culture: The importance of context in regulation of function. *Encyclopedia of Molecular Cell Biology and Molecular Medicine (EMCBMM)*. (In press, 2005)

DW Knowles, D Sudar, C Bator-Kelly, MJ Bissell, and **SA Lelièvre**. "Automated local bright feature image analysis of nuclear protein distribution identifies changes in tissue phenotype". (Submitted)

PC Abad, J Lewis, IS Mian, S Badve, J Xie, and **SA Lelièvre**. "The nuclear apparatus protein NuMA impacts higher order chromatin structure in mammary epithelial morphogenesis". (To be submitted)

G Chandramouly, PC Abad, and **SA Lelièvre**. "Restoration of higher order nuclear structure characteristic of phenotypically normal differentiation is critical for the reversion of tumor phenotype." (To be submitted)

Research manuscripts in preparation for submission in the Summer:

C Plachot, H Adissu, E Asem, and **SA Lelièvre**. "The use of natural and artificial matrices for physiologically relevant culture of non-neoplastic and neoplastic breast epithelial cells"

H Adissu, S Clark, M Johnson, S Devarakonda, and **SA Lelièvre**. "Phenotypically normal mammary epithelial cells promote survival and proliferation of tumor cells via paracrine influence"

(* indicates non-peer reviewed)

PARTICIPATION IN RESEARCH TRAINING AT PURDUE UNIVERSITY

'Administrative' member in Graduate Programs: member of the graduate committee of the Department of Basic Medical Sciences (BMS); member of the admission committee of the Interdisciplinary PULSe Program
'Participatory' member in the BMS training program, the Chromatin and Regulation of Gene Expression PULSe training program and the Integrated Molecular Signaling and Cancer Biology PULSe training program

Research trainees in the laboratory

Postdoctoral trainee: Cedric Plachot (10/2001-ongoing), Purdue Cancer Center Fellowship

Graduate trainees:

Patricia Abad (Fall 2001-Summer 2003) degree obtained: M.S.

Patricia Abad (Fall 2003- ongoing) Ph.D. program; Purdue Doctoral Fellowship

Gurushankar Chandramouly (Fall 2001- expected graduation Fall 2005) Ph.D. program, Andrews Fellowship and Purdue Research Foundation Fellowship

Hibret Adissu (Fall 2002-ongoing) Ph.D. program, Ross Fellowship and ATF Fellowship

Undergraduate, veterinary student, high school student trainees (research taken for credit or as part of research programs with fellowships from DOD, Howard Hughes, Merk Merial, MARC/AIM, and Purdue Cancer Center training programs):

- (1) Tushendar Rasiah (Senior Undergraduate; Biological Sciences, international exchange), Spring 2001
- (2) Rosemary Ruffin (Junior Undergraduate; Food Science/Biochemistry), Spring semester and Summer 2001
- (3) Sara Clark (Sophomore, Veterinary School, **Merk Merial Fellow**), Summer 2001
- (4) Katie Gumble (Junior Undergraduate, Biological Sciences), Fall 2001
- (5) Zoltan Metlagel (Senior Undergraduate, Biochemistry), Fall 2001, Spring and Summer semesters 2002, [Carroll County Cancer Association/Indiana County **Cancer Societies Fellow**]- HONORS program
- (6) Sheela Devarakonda (Sophomore, Veterinary School, **Merk Merial Fellow**), Summer 2002

(7) Katharine Turner (Pharmacy student, **DOD Fellow**), Summer 2002

(8) Jason Lewis (Junior Undergraduate, Microbiology), 2003, including summer as **DOD Fellow**

(9) Aniysha Nelpurakal (Junior Undergraduate, Biology), Spring 2003 and Summer 2003 as a **Howard Hughes Fellow**, Fall 2003 and Spring 2004- HONORS program.

(10) Monica Johnson (Sophomore, Veterinary School, **Merck Merial Fellow**), Summer 2003

(11) Dania Jaara (Junior Undergraduate, Biological Sciences), 2004, including Summer as a **DOD Fellow**

(12) Lesley Chaboub (Senior Undergraduate, Biological Sciences), international exchange, Spring 2004

(13) Ashwini Pai (Freshman Undergraduate, Biological Sciences), Spring 2004

(14) Beverly Basham (Sophomore, Veterinary School, **Merck Merial Fellow**), Summer 2004

(15) Marissa Dixon (Junior Undergraduate, **MARC/AIM Fellow**), Summer 2004

(16) Eugenia Gabrielov (High School Student, Science Project), Fall 2004-Spring 2005

ACCELERATED COMMUNICATION

The C terminus of the nuclear protein NuMA: Phylogenetic distribution and structure

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ANIYSHA NELPURACKAL,¹ CAROL BATOR-KELLY,¹ AND SOPHIE A. LELIÈVRE¹

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(RECEIVED June 1, 2004; FINAL REVISION June 1, 2004; ACCEPTED July 11, 2004)

Abstract

The C terminus of the nuclear protein NuMA, NuMA-CT, has a well-known function in mitosis via its proximal segment, but it seems also involved in the control of differentiation. To further investigate the structure and function of NuMA, we exploited established computational techniques and tools to collate and characterize proteins with regions similar to the distal portion of NuMA-CT (NuMA-CTDP). The phylogenetic distribution of NuMA-CTDP was examined by PSI-BLAST- and TBLASTN-based analysis of genome and protein sequence databases. Proteins and open reading frames with a NuMA-CTDP-like region were found in a diverse set of vertebrate species including mammals, birds, amphibia, and early teleost fish. The potential structure of NuMA-CTDP was investigated by searching a database of protein sequences of known three-dimensional structure with a hidden Markov model (HMM) estimated using representative (human, frog, chicken, and pufferfish) sequences. The two highest scoring sequences that aligned to the HMM were the extracellular domains of β 3-integrin and Her2, suggesting that NuMA-CTDP may have a primarily β fold structure. These data indicate that NuMA-CTDP may represent an important functional sequence conserved in vertebrates, where it may act as a receptor to coordinate cellular events.

Keywords: nuclear mitotic apparatus protein; β 3-integrin; chordate; mammary epithelial cells; differentiation

NuMA is widely expressed in the nuclei of mammalian cells (Kallajoki et al. 1992; Tang et al. 1993; Lelièvre et al. 1998). A prominent feature of this 2101-amino-acid protein is an unusually long coiled-coil domain spanning residues 216–1700, a region similar to the coiled-coils found in structural proteins like myosin heavy chains, cytokeratins,

and nuclear lamins (Yang et al. 1992; Harborth et al. 1995). The exact function of the NuMA coiled-coil remains unknown. In contrast, the globular NuMA-NT (residues 1–215) and NuMA-CT (residues 1701–2101) flanking the coiled-coil are associated with the function of NuMA in mitosis (Compton and Cleveland 1993). On the basis of sequence analysis, a calponin homology domain has been reported within NuMA-NT and proposed as a likely interaction site for actin-related protein 1 during mitosis (Novatchkova and Eisenhaber 2002). The proximal portion of NuMA-CT contains binding sites for several proteins involved in the control of mitosis, including tubulin, LGN, and protein 4.1R (Fig. 1; Mattagajasingh et al. 1999; Du et al. 2001; Haren and Merdes 2002).

Interestingly, NuMA-CT seems also associated with functions other than mitosis. A fusion protein between NuMA lacking the distal portion of its C terminus (trun-

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Abbreviations: ESTs, expressed sequence tags; HMM, hidden Markov model; NCBI, National Center for Biotechnology Information; NuMA, nuclear mitotic apparatus protein; NuMA-CT, NuMA C terminus; NuMA-CTDP, distal portion of NuMA CT; NuMA-NT, NuMA N terminus; RAR, retinoic acid receptor; RCSB, Research Collaboratory for Structural Biology; SAM, Sequence Alignment and Modeling.

Article and publication are at <http://www.proteinscience.org/cgi/doi/10.1110/ps.04906804>.

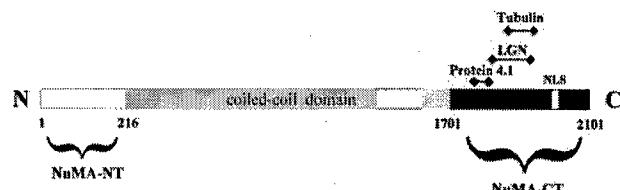


Figure 1. Protein binding regions within NuMA-CT. Numbers designate the position of amino acids. The nuclear localization signal (NLS) is indicated.

cated at amino-acid 1883) and the RAR (Wells et al. 1997) has been shown to prevent neutrophil differentiation and proposed to be involved in acute promyelocytic leukemia by altering NuMA function (Sukhai et al. 2004). In addition, introduction of antibodies directed against NuMA-CT or expression of amino acids 1965–2101 in human mammary epithelial HMT-3522 S1 cells disrupted the distribution of NuMA and altered chromatin structure and differentiation into growth-arrested mammary epithelial tissue structures (acini) obtained on culture in Matrigel (Lelièvre et al. 1998; P. Abad and S. Lelièvre, unpubl.). Thus, NuMA-CT appears to play a role in differentiation. To further decipher the function of NuMA, we have investigated the phylogenetic distribution and structure of NuMA-CTDP.

Results and Discussion

Proteins with a NuMA-CTDP-like region are present in a phylogenetically diverse set of organisms

To date, NuMA-like proteins have been identified in *Homo sapiens* (human), *Mus musculus* (mouse), *Rattus norvegicus* (rat), *Bos taurus* (cow), and *Xenopus laevis* (frog). To identify proteins possessing NuMA-CTDP-like regions, we used human NuMA residues 1915–2095, designated hNuMA-CTDP, as the protein query for TBLASTN searches against databases of genomic sequences or ESTs. Both the region missing in the NuMA-RAR fusion protein (Wells et al. 1997) and a region believed to be involved in the control of mammary differentiation (P. Abad and S. Lelièvre, unpubl.) are included within hNuMA-CTDP. We found sequences with statistically significant similarity to hNuMA-CTDP in *Sus scrofa* (pig), *Gallus gallus* (chicken), *Danio rerio* (zebrafish), and *Takifugu rubripes* (pufferfish; Fig. 2). The putative pig and chicken family members were translated ESTs. For pufferfish, part of *T. rubripes* genomic scaffold 307 contained short peptide matches in the -1 frame. This scaffold sequence and the Web version of GENSCAN (Burge and Karlin 1997) were used to identify an 865-amino-acid open reading frame containing the hNuMA-CTDP-like region at its C terminus. The zebrafish hNuMA-CTDP relative was identified in a similar manner using the

February 2004 Ensembl assembly of the genome (<http://www.ensembl.org/Danio rerio>). Given the techniques and tools available currently, we found no convincing evidence for hNuMA-CTDP family members in three other metazoa with fully sequenced genomes (*Caenorhabditis elegans*, *Drosophila melanogaster*, and *Arabidopsis thaliana*). However, we did find a short match to hNuMA-CTDP in a *Ciona intestinalis* genomic scaffold. *C. intestinalis* is considered to be one of the earliest chordates because although the larval stage has a notochord, it is lost in the adult stage. These observations suggest a relatively broad phylogenetic distribution for members of the NuMA-CTDP family, with convincing evidence for their presence in vertebrates (mammals: human, mouse, rat, pig; amphibians: frog; birds: chicken; and teleost fish: zebrafish and pufferfish) and a possibility that NuMA itself might be restricted to the chordate lineage.

NuMA-CTDP family members may possess a fold similar to the extracellular domain of human $\beta 3$ -integrin

To gain insight into the structure and thus function of NuMA-CTDP, we sought to predict a possible three-dimensional structure for the NuMA-CTDP family. There were no matches when hNuMA-CTDP was used as the query for an NCBI Conserved Domain Database Search. Therefore, we estimated an HMM from a set of diverse NuMA-CTDP sequences, hNuMA-CTDP plus its frog, chicken, and teleost relatives (HsNuMA1, XINuMA, GgEST, and FrORF, as shown in Fig. 1) using the SAM System (<http://www.soe.ucsc.edu/research/compbio/sam.html>). The resulting NuMA-CTDP HMM was used to search a database of protein sequences of known three-dimensional structure provided by the RCSB (<http://www.rcsb.org>). The highest scoring protein of the ~55,000 sequences in the February 2004 release from the RCSB was human $\beta 3$ -integrin (1m1x). The sequence of this region of $\beta 3$ -integrin is highly conserved in human $\beta 1$ - and $\beta 6$ -integrins. The next highest scoring protein was another membrane protein, oncoprotein receptor tyrosine kinase Her2 (1n8y). Although the scores of 1m1x and 1n8y against the NuMA-CTDP HMM were not statistically significant (E values above 1.0), the results are of biological interest because the regions of similarity correspond to largely β extracellular domains that regulate protein interactions and are involved in signaling (Borges et al. 2000; Hynes 2002). These results suggest that the three-dimensional structure of NuMA-CTDP might be primarily β , and that this region could be a site of intermolecular interaction(s).

If NuMA-CTDP is a site of interaction with other proteins, then by analogy with $\beta 3$ -integrin, potential binding partners may include proteins located at the plasma membrane. More specifically, this observation suggests that in addition to its known location in the nucleus, NuMA could

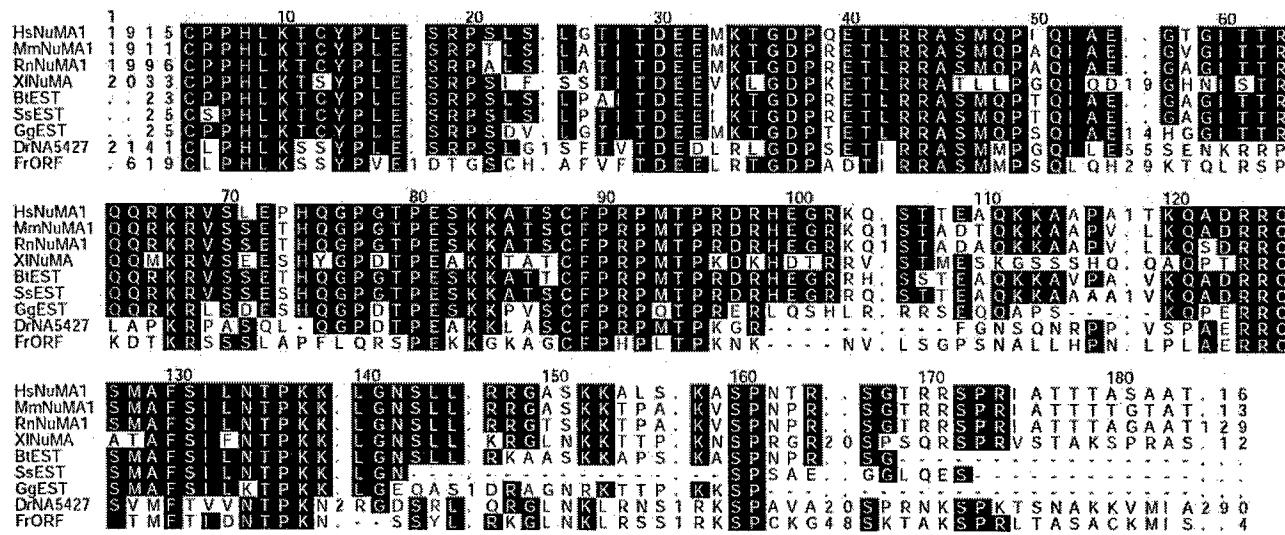


Figure 2. Multiple sequence alignment of the C-terminal non-coiled-coil segment of NuMA family members. The sequences shown are HsNuMA1 (*Homo sapiens* NuMA; databank code NP_006176; human), MmNuMA1 (*Mus musculus* NuMA; NP_598708; mouse), RnNuMA1 (*Rattus norvegicus* NuMA; XP_218972.2; rat), XInuMA (*Xenopus laevis* NuMA; T30336; frog), BtEST (*Bos taurus* EST; BI680620; GI: 15633534; cow), SsEST (*Sus scrofa* EST; BX67330.1, GI: 37986836; pig), GgEST (*Gallus gallus* EST; ChEST630g3, [<http://chick.umist.ac.uk/>] accession no.: 603737612F1; chicken), DrNA5427 (*Danio rerio* Ensembl GENSCAN predicted peptide; NA5427.1.260.43185; zebrafish), and FrORF (*Takifugu rubripes* GENSCAN predicted peptide in *Fugu* scaffold 307; pufferfish). Positions conserved in at least five of the nine sequences are highlighted; the number of residues not displayed explicitly is listed.

be present at or near the cell membrane. Indeed, full-length NuMA is observed in the cytoplasm of cells expressing oncogenic fusion protein NuMA-RAR (Hummel et al. 2002). Furthermore NuMA binds to protein 4.1R, a protein that participates in tethering the cytoskeleton to the plasma membrane in addition to being located at the poles of the mitotic spindle (Matajagasingh et al. 1999). Although NuMA could be detected in the cytoplasm of S1 cells differentiated into acini, as seen on electron micrographs (not shown), it was not located at the cell membrane and was not present in preparations of crude membrane extracts that include the plasma membrane, Golgi apparatus, and rough endoplasmic reticulum (Fig. 3). To rule out the possibility that the absence of NuMA in membrane fractions was a characteristic of acinar differentiation, we performed the same analysis using collagen I culture of S1 cells, which induces the formation of incorrectly polarized acini, and HMT-3522 T4-2 malignant cells derived from S1 cells. T4-2 cells form disorganized tumor nodules when cultured in Matrigel (Weaver et al. 1997). NuMA was still absent from crude membrane fractions prepared under these conditions (Fig. 3). Thus, it seems unlikely that NuMA interacts with proteins at the cell surface.

The presence of NuMA-CTDP family members in vertebrates including species with extreme phylogenetic separation (i.e., mammals and teleost fish), together with their likely absence in invertebrates, suggests that NuMA-CTDP may have a highly conserved vertebrate-specific role. The investigation of the three-dimensional structure of NuMA-

CT is a key tool to help further decipher NuMA functions. It has been proposed that the proximal portion of NuMA-CT, which contains binding sites for tubulin and LGN and is important for the function of NuMA during mitosis, may consist of an α helix structure (Haren and Merdes 2002). In our study, we describe a potential structural similarity between NuMA-CTDP and the extracellular domain of $\beta 3$ -integrin, which has a largely β structure. The β strand is considered to bring maximum exposure for ligand binding and may lead to intermolecular linkage (Chow et al. 2004). This secondary structure has been identified as a critical recognition element in physiological processes (Glenn and Fairlie 2002) and is present in numerous proteins involved in signal transduction. These data suggest that NuMA may also function to orchestrate cellular events.

Given that NuMA has a role in differentiation (Lelièvre et al. 1998; Sukhai et al. 2004), one hypothesis is that NuMA-CTDP may be associated with the control of gene expression. This hypothesis is supported by the fact that expression of NuMA truncated at its C terminus and antibodies directed against NuMA-CT induce alterations in chromatin organization (Gueth-Hallonet et al. 1998; Lelièvre et al. 1998). Recently proteins bearing actin-binding domains have been proposed to play a critical role in the control of gene expression by providing a structural framework that facilitates and integrates molecular cross-talk within the nucleus (Shumaker et al. 2003). Thus, with its calponin homology domain at the N terminus and a possible β structure for NuMA-CTDP, NuMA may provide a structural

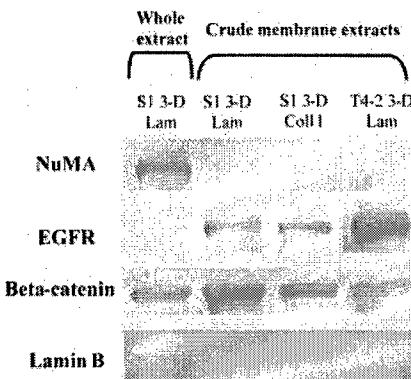


Figure 3. NuMA is absent from the plasma membrane of HMT-3522 cells. Non-neoplastic S1 and malignant T4-2 HMT-3522 cells were cultured for 10 d in the presence of Matrigel (S1 3-D Lam and T4-2 3-D Lam) or collagen I (S1 3-D Coll I). Western blots of whole-cell extracts and crude membrane fractions are shown for NuMA, plasma membrane region markers epidermal growth factor receptor (EGFR) and β -catenin, and nuclear marker lamin B.

platform for the transduction and coordination of signals involved in the regulation of gene expression.

Materials and methods

Sequence analysis

We used *H. sapiens* NuMA residues 1915–2095 as the query for TBLASTN searches against the complete genomes or ESTs (protein vs. translated DNA) of *B. taurus*, *S. scrofa*, *R. norvegicus*, *M. musculus*, *G. gallus*, *X. laevis*, *T. rubripes*, *D. rerio*, *C. intestinalis*, *C. elegans*, *D. melanogaster*, and *A. thaliana*. Statistical modeling, alignment, and database searching were performed using HMMs as implemented in the SAM software (Hughey and Krogh 1996). TBLASTN and all other programs used in this work were run with default parameter settings.

Cell culture

HMT-3522 nonneoplastic (S1) and malignant (T4-2) cells were cultured in H14 medium (Weaver et al. 1997). To induce differentiation into acini and the formation of tumor-like nodules, we cultured S1 and T4-2 cells, respectively, for 10 d on 40 μ L/cm² Matrigel (BD Biosciences)-coated surfaces in the presence of culture medium containing 5% Matrigel. Collagen I culture of S1 cells was performed as previously described (Lelièvre et al. 1998).

Crude membrane extract preparation

Cellular structures were collected as described earlier (Lelièvre et al. 1998) and crude membrane fractionations were performed according to standard procedures. Briefly, after dissociation of nuclei and cytoplasmas, cytoplasmas were deposited on top of 10 mL of a buffer containing 10 mM HEPES (pH 7.4), 1 mM ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid, and 2 mM MgCl₂ and centrifuged (4°C, 114,000g) for 75 min to separate

cytoplasmic (supernatant) from crude membrane (pellet) proteins. Crude membrane fractions were analyzed by Western blot using antibodies against lamin B and NuMA (clone 204.4; Oncogene Research Products) and β -catenin and epidermal growth factor receptor (BD Transduction Laboratories).

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Chapter 2

Novel Directions in Tumour Biology: From Basement Membrane-Directed Polarity to DNA Methylation

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2.7 References

2.1 Abstract

It is now well established that dynamic interactions between cells and their microenvironment are essential for the regulation of tissue function, cell behaviour, and gene expression. Notably the influence of extracellular matrix proteins on structural and functional epithelial tissue differentiation and normal epithelial cell behaviour has been linked to specific interactions between basement membrane-related extracellular matrix proteins and their cell membrane receptors. The importance of the basement membrane in tissue polarisation, a critical parameter of tissue differentiation and cell survival, was clearly demonstrated using three-dimensional cultures of epithelial cells that mimic physiologically relevant conditions. A major advancement in the understanding of the epigenetic regulation of gene expression has been obtained thanks to the identification of several molecular systems involved in chromatin remodelling. These discoveries have enabled biologists to begin deciphering the mechanisms by which extracellular matrix signalling regulates gene expression. In this chapter, we will review the fundamental processes of basement membrane-directed polarisation and chromatin remodelling and discuss how alterations in these processes may be associated with tumour development. An overview of novel therapeutic strategies targeted to the regulation of cell-extracellular matrix interactions and chromatin remodelling factors in cancer cells will also be presented.

2.2 Introduction

The identification of novel targets for cancer therapy relies on the understanding of the mechanisms that direct tumour behaviour. This review will develop two aspects of tumour biology that have brought considerable potential for understanding tumour development and cancer progression, and discuss their relevance for cancer therapy. The first aspect focuses on the relationship between tumour cells from epithelial origin and their microenvironment and more particularly the basement membrane, a specific type of extracellular matrix (ECM). The basement membrane acts as a signal transducer to epithelial cells, an organiser of cellular compartmentalisation and tissue polarisation, and a physical barrier between epithelial structures and the surrounding stroma. Changes in cell-basement membrane interaction occur early during tumour development and increasing alterations between basement membrane organisation and basement membrane receptors at the surface of tumour cells may account for significant modifications in tumour cell behaviour during cancer progression.

The second feature of tumour biology we are addressing in this chapter is related to epigenetics, which refers to the control of gene expression without alterations in DNA sequence. It has become increasingly evident that beside numerous examples

of mutations and other DNA aberrations found in the genome of tumour cells, a number of unaltered genes coding for key regulatory proteins are simply turned on or off. Most often alterations in the expression of genes are the consequences of chromatin remodelling that switches gene promoters between open and closed conformations. Two mechanisms involved in chromatin remodelling, the ATP-dependent SWI/SNF modulatory complex and post-translational histone modifications, have been deciphered. These mechanisms are influenced by DNA methylation, which chemically modifies DNA and hence regulates the recruitment of the machinery necessary for chromatin remodelling. Understanding how genes are controlled locally has opened new possibilities for the implementation of strategies aimed at altering gene expression, and notably the expression of genes involved in proliferation control, in cancer.

Finally, in a third section of the chapter, we will discuss promising directions in tumour biology research by exploring the connection between basement membrane signalling and chromatin remodelling. In each chapter section a particular aspect of tumour biology will be discussed in comparison with the normal situation, since as it was proposed more than two decades ago [1], the best way to understand how cells go awry is to first understand how cells behave normally.

2.3 Cell-Basement Membrane Interactions during Tumour Progression

More than 80% of cancers originate from epithelial tissue and one of the earliest modifications in the neoplastic epithelium is the alteration of tissue polarity. Tissue polarisation is a characteristic of epithelial differentiation and is accompanied with the compartmentalisation of proteins along the polarisation axis (Figure 2.1).

This phenomenon induces an asymmetrical intracellular organisation in which different subcellular areas are morphologically or functionally distinct. Tissue polarity is responsible for the appropriate function of the epithelial tissue. For instance it influences cell proliferation and directs secretion vectorially into the lumen in breast, salivary, or pancreatic glandular structures [2, 3, 4] and also regulates appropriate cell assembly in the epidermis [5]. Typically polarisation is initiated by the deposition of a continuous basement membrane against what then becomes the basal plasma membrane of epithelial cells. Basement membrane deposition triggers the redistribution of receptors of basement membrane components to the basal cell membrane, while proteins involved in cell-cell junctions redistribute to cell membranes in contact with other cells. The predominant and most studied receptors of the basement membrane are the integrins that belong to a superfamily of proteins capable of heterodimerization. Unravelling alterations in both basement membrane and integrin organisation in tumours has considerably improved our understanding of the mechanisms involved in tumour progression and resistance to treatment.

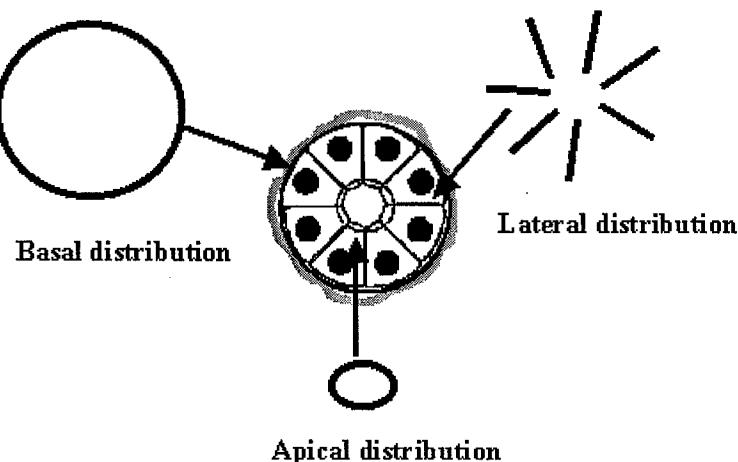
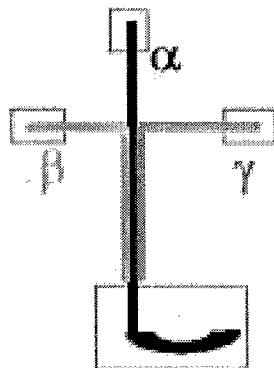


Figure 2.1

Compartmentalisation of proteins along the polarisation axis. The centre of the figure represents a polarised glandular structure, in which one layer of epithelial cells is organised around a lumen (cell nuclei are drawn as black filled-circles). Polarisation is induced by the deposition of a continuous basement membrane (in grey) that delineates the external side of the glandular structure. Upon basement membrane deposition, cell membrane proteins redistribute to different compartments: certain proteins are only found at basal cell membranes (basal distribution), others relocate to cell-cell contacts (lateral distribution), while certain types of proteins concentrate to the cell membranes that delineate the internal lumen (apical distribution).

2.3.1 The Roles of Basement Membrane-Integrin Interaction in Normal Tissue

The basement membrane is composed of an organised lattice of fibrous proteins, including nidogens, laminins and collagen IV, and proteoglycans that make contact with the cell membrane via specific receptors [6, 7]. Laminins are among the main constituents of basement membranes. These heterotrimeric glycoproteins are composed of heavy α , light β , and light γ chains held together by disulphite bonds (Figure 2.2). To date 11 different chain isoforms have been identified [7] and have been described to be expressed in a tissue-specific and developmentally regulated

**Figure 2.2**

Structure of laminin. Three types of chains (α , β , and γ) organised cruciformly are involved in laminin formation. Binding sites for major laminin receptors (integrins) are indicated by a box.

manner. All major types of basement membranes contain at least one of the laminin variants. For instance, in the mammary gland laminin 1, 5, 9, and 10 are present and are mainly in contact with myoepithelial cells that surround luminal epithelial cells. Laminins are also in contact with a portion of the luminal cell surface [8]. In the epidermis, keratinocytes are in continuous contact with laminin 5 and 1 [9]. It was recently shown using lung organotypic cultures that in order to mediate polarisation laminin must be polymerised and that polymerisation is mediated by the outer globular region of its β chain [10].

The major receptors of fibrous basement membrane molecules belong to the integrin family of proteins. The term integrin was chosen because these molecules are integral membrane proteins (Figure 2.3) and play a major role in the integration of functions between the ECM and the cytoskeleton [11]. They act as noncovalently associated α/β heterodimers that were originally thought to mediate exclusively cell-ECM interactions in multicellular organisms. Integrins possess a cytoplasmic domain that participates in cellular signalling via its ability to associate with and activate signal transduction pathways, and to connect with the cytoskeletal network. The extracellular domain of integrins binds to ECM proteins including fibronectin, collagen, nidogen, and laminin [12]. The interaction between integrins and laminin seems to be largely determined by the α chain of laminin. However, integrins can also bind counter receptors on adjacent cells. This is illustrated by the localisation of $\beta 1$ and αv integrins to cell-cell boundaries. Such a localisation has been proposed to participate in the maintenance of polarity by integrating or supporting the role of other cell-cell adhesion molecules [13, 14].

To date 18 α -integrin chains and 8 β -integrin chains have been identified in

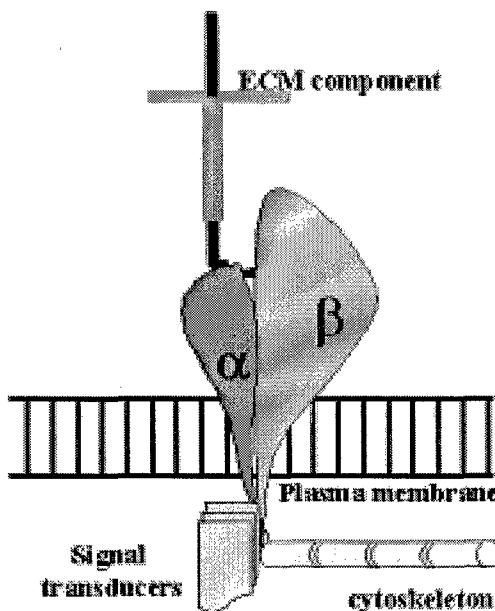


Figure 2.3

Integrin heterodimers and signal transduction. Upon the binding of their extracellular domains to specific ECM components (e.g., a cruciform laminin is represented in this image) α and β integrin heterodimers interact, via their intracellular domains, with signal transducers and elements of the cytoskeleton.

vertebrates. In addition, for most chain types, several isoforms can be generated by alternative mRNA splicing. Binding specificity is determined by both the conformation of individual α and β chains and the cellular context [15, 16]. $\alpha 1/\beta 1$, $\alpha 2/\beta 1$, $\alpha 3/\beta 1$, $\alpha 6/\beta 1$, $\alpha 7/\beta 1$, $\alpha 9/\beta 1$, $\alpha v/\beta 3$, $\alpha v/\beta 5$, $\alpha v/\beta 8$, and $\alpha 6/\beta 4$ have all been reported to bind laminin. Among those, $\alpha 6/\beta 1$, $\alpha 7/\beta 1$, and $\alpha 6/\beta 4$ are viewed almost exclusively as laminin receptors. The $\beta 4$ subunit is unique among integrins because it possesses a distinctively large cytoplasmic domain (1000 amino-acids instead of 50 amino-acids) and encompasses fibronectin type III repeats that bring elasticity and allow resistance to mechanical forces [17]. The first pair of repeats has been shown to mediate $\beta 4$ -integrin unique association with intermediate filaments via an interaction with plectin [18]; other integrins are mainly connected to the actin network.

$\alpha 6/\beta 4$ -integrins are localised to the basal plasma membrane of polarised epithelial cells where they preferentially interact with laminin 5. Their role in polarisation is exemplified by their specific localisation to hemidesmosomes, the ultrastructures that maintain adhesion of the epithelium to the underlying basement membrane

and are critical for the induction and maintenance of tissue polarity [19]. The role of $\alpha 6/\beta 4$ integrins in tissue polarity has been well demonstrated thanks to the use of three-dimensional (3D) culture systems that reproduce glandular differentiation [2, 20–23] and epidermis formation [5] of normal and nonmalignant cells placed in contact with a reconstituted basement membrane. Human primary mammary luminal epithelial cells cultured within collagen-I gels instead of reconstituted basement membrane arrested growth and formed 3D cell clusters; however, they did not polarise, as shown by the deposition of milk precursors at the basal rather than the apical pole of the cell clusters and the absence of $\beta 4$ -integrin [8]. Subsequent addition of laminin 1 was sufficient to restore polarity and $\beta 4$ -integrin localisation at basal cell membranes. In another study, $\beta 4$ -integrin blocking antibodies were able to suppress polarity in breast glandular structures and induce cells to proliferate [24].

$\beta 1$ -integrins have been involved in polarity by acting directly on the assembly of the basement membrane. They mainly localise to regions of cell-cell interaction; however, certain dimers, like $\alpha 3/\beta 1$ are also located to the basement membrane area. Expression of $\beta 1$ -integrin lacking its extracellular domain induced poor lactation performances in transgenic mice. Alterations in the function of the mammary gland were accompanied with an abnormal accumulation of laminin and $\beta 4$ -integrin at the lateral surface of luminal epithelial cells [25].

Another important function of integrin-mediated polarity is the maintenance of cell survival in a growth-arrested state. This function was demonstrated by interrupting $\beta 1$ -integrin binding using function-blocking antibodies in 3D mammary cultures [26] and expressing truncated $\beta 1$ -integrin in mice [25]. Both manipulations induced apoptosis in mammary luminal epithelial cells. Furthermore, adhesion-mediated activation of $\beta 4$ -integrin was shown to trigger phosphorylation of Akt and Bad, two transducers involved in the inhibition of apoptotic pathways [27]. However, the authors suggested that cell survival might result from integrin involvement in the maintenance of cell adhesion rather than from a direct interaction between a particular integrin and mediators of apoptotic pathways.

2.3.2 Alteration of Polarity in Cancer

Originally, reports described that tumour progression was paralleled by changes in basement membrane organisation and hence might be accompanied by alterations in polarity. Discontinuous laminin has been observed in pancreatic acinar carcinoma [28] and other reports have indicated that well-differentiated gastric adenocarcinomas have a discontinuous but thick basement membrane, while increasing loss of differentiation is associated with the production of thinner and fragmentary, or even totally disorganised, basement membranes [29]. Similarly, organotypical cultures of mouse epidermal invasive tumour cells revealed that classical basement membrane components are expressed but there is no formation of a structured basement membrane, except in some areas of well differentiated tumours [30]. The presence of intact basement membrane in certain tumour regions was intriguing since it sug-

gested that tumour cells in these areas might maintain polarity. The consequence of the presence of polarised tumour cells for tumour behaviour was recently unravelled. Indeed maintenance of $\beta 4$ -integrin-mediated polarity may drive resistance to apoptosis normally induced by cytotoxic drugs. This possibility is illustrated by recent work showing that the expression of altered $\beta 4$ -integrin in nonmalignant human breast epithelial cells is accompanied with impaired polarisation and increased sensitivity to cytotoxic drugs. Whereas, ligation of $\beta 4$ -integrins in tumour cells and induction of hemidesmosome formation initiate resistance to apoptosis induction via NFkB activation [31].

The loss of polarity usually seen in many tumours can easily be linked to the presence of an incomplete basement membrane. In mammary cancer, this hypothesis is based on the fact that mesenchymal cells that usually produce a lot of a laminin rapidly disappear during tumour progression. Although tumour cells can produce a number of basement membrane components, they make minimal amounts of a laminin, which results in the formation of incomplete laminin [32]. In addition the increase in matrix metalloprotease (MMP) activity [33], and notably gelatinase activity, in the vicinity of tumour cells may be responsible for the cleavage of basement membrane components and thus contribute to the alteration of polarity, as suggested by MMP2-mediated cleavage of the $\gamma 2$ chain of laminin 5 in certain tumours [34].

Alterations in basement membrane receptors also contribute to the loss of polarity in tumours. Malignant development is often associated with defective hemidesmosomal structures [35]. $\alpha 6/\beta 4$ integrins may still be expressed but they are not localised to hemidesmosomes. Depending on the type of neoplastic lesion, the alteration of polarity may be accompanied by either an increase in $\alpha 6/\beta 4$ integrin expression (e.g., squamous cell carcinoma) or a decrease in $\alpha 6/\beta 4$ integrin expression (e.g., basal cell carcinoma) [36]. Alterations in $\alpha 2/\beta 1$ and $\alpha 3/\beta 1$ integrins are also observed, as shown by their pericellular distribution instead of typical cell-cell localisation in epidermal carcinoma [5]. A change in the ratio between $\beta 4$ and $\beta 1$ -integrins have been reported between malignant, nonpolarised HMT-3522 mammary epithelial cells and their compared to their nonmalignant counterpart. Interestingly polarity and glandular differentiation could be reinduced in these tumour cells using function blocking $\beta 1$ -integrin antibodies, indicating that modifications in the dominant integrin pathway may also be involved in the loss of polarity associated with tumour development [24].

Interestingly, once released from their participation in tissue polarity, integrins become involved in tumour behaviour. $\alpha 6/\beta 4$ -integrins may redistribute to actin-rich cell protrusions during migration of carcinoma cells [37] and may promote invasion via activation of phosphoinositide 3-kinase [38]. The same integrin dimer has also been shown to stimulate chemotactic migration, a key component of invasion [39]. Similarly $\alpha 6/\beta 1$ integrins may promote adhesion, migration, and survival of tumour cells [24, 40, 41]. These cancer-related functions may be mediated either by the appearance of a splice variant, as reported for the $\alpha 6$ -integrin subunit [42], the binding of truncated laminin (e.g., peptides derived from the laminin α chain) [43], or abnormal clustering, as shown for $\alpha 6/\beta 1$ -integrins [44]. In addition, the formation of integrin clusters in invadopodia may serve as docking sites for MMPs and hence

allow focal proteolysis of the surrounding matrix [45].

Alterations in basement membrane deposition and integrins are obvious illustrations of the loss of polarity. However, more subtle alterations in cell and tissue polarity appear very early during tumour development. In carcinoma *in situ*, a basement membrane still delineates the basal surface of the external cell layer, but the central lumen has been completely filled by tumour cells that are not in contact with the basement membrane (Figure 2.4). This phenomenon was reproduced in culture by activating erb2 receptor, a member of the epidermal growth factor (EGF) signalling pathway, in preformed mammary glandular structures [46]. This study suggests that constitutive activation of EGF-related signalling pathways may contribute to tumour cell survival upon the loss of basement membrane contact. In addition, this survival could be mediated by the loss of function of proteins involved in positioning control, that normally direct the requirement for basement membrane attachment. For instance expression of the tumour suppressor protein Dab2 is frequently lost in ovarian and breast cancers. Reexpression of Dab2 in ovarian and breast tumour cells was shown to induce cell death. However, cell death could be prevented by the attachment of tumour cells to a basement membrane [47].

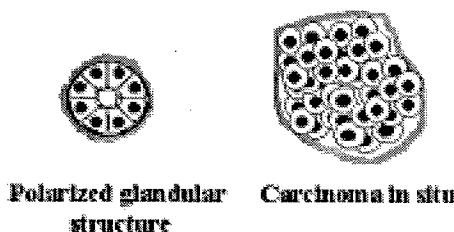


Figure 2.4

Epithelial cell organisation in normal glandular structures and carcinoma *in situ*. A section through a normal and fully polarised (baso-apical polarisation) glandular structure shows the central lumen (left image), while a section through a carcinoma *in situ* surrounded by a continuous basement membrane shows a mass of tumour cells; the basement membrane is in light grey.

2.3.3 Novel Anti-Cancer Strategies Based on Cell-ECM Interaction

The better understanding of the role of the basement membrane and its receptors in the regulation of normal and tumour cell behaviours has led to the development of both general and specific anti-cancer strategies. A strategy currently tested in clinical trials is to prevent the expression of MMPs that degrade the basement membrane and stromal ECM and are found activated in most tumour types investigated so far. Broad-spectrum MMP inhibitors produced initially have a structure that imitates collagen structure. They act by chelating the zinc ion present at the active site of MMPs. These broad range inhibitors have shown promising results in clinical trials and have been proposed to be used in adjuvant treatment or maintenance therapy following response to usual cytotoxic drugs [48]. They are also being investigated for use in refractory cancers [49]. It would certainly be an advantage, in order to reduce the extent of side effects and get a higher response rate in certain types of cancer, to use inhibitors targeted to specific MMPs. For instance, inhibitors specific of basement membrane proteases MMP-2 and MMP-9 have been recently developed and have been shown to prevent tumour growth and invasion in xenograft-bearing animals [50]. Other possibilities for more specific targeting of defined MMPs could include the use of ribozymes that block MMP expression. Such a system has been under investigation *in vitro* for MMP-9 [51]. Nevertheless, caution should be used with inhibitors of MMP activity that act principally in the cell's microenvironment. Tumour cells may not be directly targeted (with the exception of the use of ribozymes) and might easily develop counter-actions that would still permit tumour progression. Therefore MMP inhibitors may only be useful in combined therapy with agents that directly act on tumour cells. Novel approaches have also been developed to inhibit tumour invasion by blocking or down-regulating integrins involved in tumour cell adhesion. FTY720, an immunosuppressive agent, significantly prevents tumour cells adhesion and migration on ECM by inducing a decrease in integrin expression via an unknown mechanism [52]. Another compound, Contortrostatin, a homodimeric disintegrin (a soluble integrin ligand capable of disrupting cell-matrix interactions), has been shown to prevent cell invasion through an artificial basement membrane and significantly inhibit ovarian cancer dissemination in a nude mice model [53].

None of these therapeutic approaches have yet been developed to modulate basement membrane-related cellular polarity. However, in light of the results reported above and showing that tumour cells may resist apoptosis induction by cytotoxic drugs via $\beta 4$ -integrin-mediated polarity, a promising strategy may be to block this pathway in certain tumours. A possible approach could be to use recombinant human antibodies specific for laminin. Such antibodies have been recently developed and shown to reduce tumour cell attachment to laminin [54]. The authors have proposed that these antibodies might have a potential application in cancer therapy. Nevertheless, like for many therapeutic tools, the difficulty will be to intervene only at the tumour cell level and not the normal tissue level.

2.4 Chromatin Remodelling and Cancer

Chromatin is defined as the organised assembly of DNA, histone and nonhistone proteins. The basic repeating units of chromatin, referred to as nucleosomes, are composed of 147 base pair DNA sequences wrapped around an octamer of four different histone proteins (H2A, H2B, H3, and H4). This nucleosomal configuration can be further condensed (e.g., by histone H1) to form “heterochromatin.” Chromatin condensation prevents DNA accessibility to transcription factors and the transcriptional machinery, and ultimately leads to the repression of gene transcription. In contrast, decondensed chromatin fibres form an open chromatin or “euchromatin” that may be associated with active gene expression. The reorganisation of chromatin between open and closed conformations is referred to as “chromatin remodelling” [55].

Different systems are involved in the control of chromatin structure:

- the ATP-dependent chromatin remodelling complex switching/sucrose non-fermenting (SWI/SNF) which mediates the sliding of nucleosomes along DNA [56],
- protein complexes that induce covalent histone modifications, including acetylation, phosphorylation, and methylation [57].

In addition, there is growing evidence to show that the recruitment of chromatin remodelling complexes is influenced by DNA methylation, an important epigenetic mechanism of gene silencing [58]. Alterations in the function or the recruitment of chromatin remodelling complexes are associated with improper chromatin conformation and subsequent dysregulation of gene expression that seem to play a critical role in tumour development and progression [59].

2.4.1 The SWI/SNF ATP-Dependent Chromatin Remodelling Complex

The SWI/SNF complex is a multiprotein assembly (8-12 proteins) that remodels chromatin structure in an ATP-dependent manner [56].

Mechanisms by which SWI/SNF influence chromatin conformation are intricate and not yet fully understood. On one hand, SWI/SNF activity can mediate the formation of an open chromatin by altering individual nucleosomal structures or acting in concert with other chromatin remodelling factors to decondense higher-order chromatin fibres [56] (Figure 2.5). On the other hand, when associated with other chromatin remodelling factors such as the histone deacetylase Sin3, SWI/SNF activity may also be involved in inactive chromatin conformation and subsequent gene silencing, [60] (Figure 2.5). Therefore the molecular context at the level of a specific

gene promoter is likely to play a critical role in determining whether SWI/SNF will participate in closing or opening chromatin.

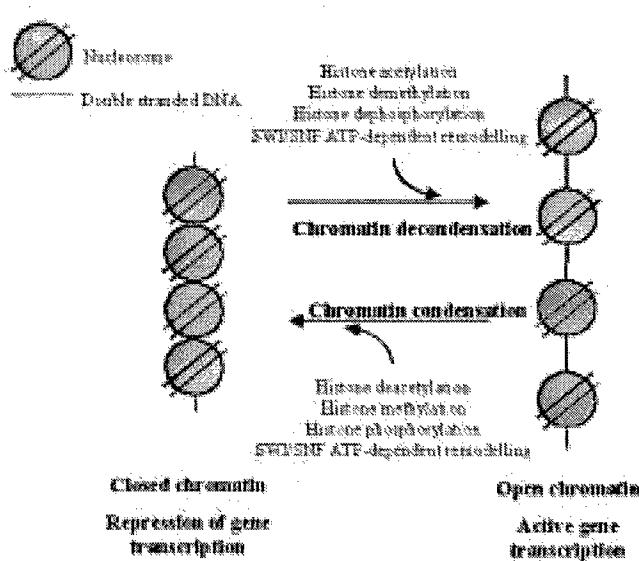


Figure 2.5

Regulation of chromatin conformation. Compaction of nucleosomes and formation of higher order chromatin folding is associated with the repression of gene transcription. Histone deacetylation induced by HDACs and SWI/SNF activity may act separately or in concert to mediate chromatin compaction. Histone methylation and phosphorylation may also provide chromatin condensation. Histone acetylation induced by HATs, histone dephosphorylation, and histone demethylation lead to open chromatin conformation, which may be associated with active gene transcription. Depending on the composition of the multiprotein complex, SWI/SNF activity may also be involved in the formation of an open chromatin.

2.4.2 Histone Modifying Enzymes

Covalent posttranslational modifications of histones are critical for nucleosome stability, and thus chromatin organisation. Acetylation/deacetylation is the most studied covalent modification of histones that participates in the control of chromatin structure. Other histone modifications may be induced by phosphorylation and methylation; although less understood these posttranslational modifications also seem to play a role in chromatin conformation. Finally, histones may also undergo ubiquitination, but the influence of such a modification on chromatin remodelling remains to be clarified.

The interaction between DNA and histones is principally mediated by the attraction between negative charges of DNA and positive charges of the lysine residues located on histone tails. The addition of an acetyl group (C_2H_3O) onto lysines is mediated by histone acetyltransferases (HATs) and neutralises the positive charges of histones, and hence may disrupt histone-DNA interactions and induce the formation of open chromatin (Figure 2.5). The reverse reaction catalysed by histone deacetylases (HDACs), increases the affinity of histones for DNA, which, in turn, triggers chromatin compaction and subsequent gene silencing [61] (Figure 2.5). HATs and HDACs are organised as multiprotein assemblies and may cooperate with ATP-dependent chromatin remodelling complexes to regulate gene transcription. This co-operation has been observed for the multi-protein assembly that forms the nucleosome remodelling and deacetylation (NuRD) complex [60].

Supplementation of histones with phosphate ion PO_4^{3-} (i.e., phosphorylation) is principally mediated by the histone H1 kinase cyclin E Cdk2 and the family of histone H3 kinases Msk/Rsk. Several studies have shown that histone phosphorylation (notably on the serine-10 residue of histone H3) may have opposite effects by promoting chromatin condensation as well as activation of gene expression. This is well illustrated for histone H3, the phosphorylation of which correlates with chromosome compaction during mitosis [62], while it is also associated with the transcription of the early-response genes *c-fos* and *c-jun* [63, 64].

Histone methylation is catalysed by histone methyl transferases (HMTs) and consists of the addition of methyl (CH_3) groups onto either arginine or lysine residues mainly located on histones H3 and H4. In contrast to acetylation, histone methylation does not change the overall charge of histone tails, but rather increases histone basicity and hydrophobicity that, in turn, enhances histone affinity for anionic molecules like DNA. Recent data suggest that histone methylation might be involved in heterochromatin assembly. Indeed, the heterochromatin-associated protein Suvar3-9 has been shown to methylate histone H3 *in vitro* [65]. Interestingly, histone methylation may also be associated with active gene transcription when preferentially targeted to acetylated histones H3 and H4 [61]. Additional support for a role of histone methylation in promoting gene expression arises from the findings of a direct interaction between HATs (e.g., CBP) and HMTs [61]. To complicate the matter further, an interplay between histone methylation and phosphorylation has also been observed.

Site-specific histone methylation on the lysine 9 residue of histone H3 prevents histone phosphorylation on serine 10. Whereas, when histone methylation is prevented upon Suvar3-9 deletion, histone H3 phosphorylation is induced and abnormal chromosome condensation follows [55]. Altogether reports suggest that one type of post-translational alteration occurring on histone probably influences the effect that additional histone alterations will have on chromatin structure.

2.4.3 Dysregulation of Chromatin Remodelling in Cancer

Cancer is characterised by dramatic changes in the pattern of gene expression. Consequently it is not surprising to find alterations in chromatin structure in tumour cells. According to the literature, these alterations are mainly due to the retargeting of chromatin modifiers to different sets of genes. However, it is unclear whether the retargeting of chromatin modifiers is a consequence of alterations in the molecules they interact with or whether initial alterations in chromatin modifiers may be sufficient to trigger the abnormal expression of specific genes.

Accumulating data strongly suggest that SWI/SNF acts as a tumour suppressor in mammalian cells. Mutations within the SNF5/INI1 components of the SWI/SNF complex have been found in several cases of rhabdoid tumour and leukaemia [66, 67], and mutations in Brg1, the ATPase subunit of the SWI/SNF complex, have been observed in prostate, lung, and breast cancer cell lines [68]. In addition, SNF5 or Brg1 knock out mice display a cancer-prone phenotype [69, 70]. A possible explanation for the tumour suppressor role of the SWI/SNF complex arose from data showing that SWI/SNF subunits interact with tumour suppressors, including Rb, BRCA1, and Myc. For example, Rb can act in concert with Brg1 to assist in gene silencing and induce growth arrest [71]. In addition, several lines of evidence suggest that functions of BRCA1 and Myc are partially dependent on the presence of SWI/SNF [72, 73]. Thus abnormal SWI/SNF may prevent proper functions of these key regulators of cell behaviour. Conversely, we cannot rule out that proteins like Rb, BRCA1, or Myc may control gene expression by directing the SWI/SNF complex to distinct promoters and hence induce chromatin remodelling. Since Rb, BRCA1, and Myc are mutated or silenced in certain cancers, alterations in their function or expression may also have repercussions on the targeting of SWI/SNF to specific DNA sequences.

Similarly to SWI/SNF, the impairment of HATs function has been linked to cancer. A well-described example is the chromosomal translocation affecting the CREB binding protein (CBP) gene, a HAT, which is associated with leukaemia [59]. This chromosomal translocation may lead to the fusion of CBP with other proteins that, in turn, modify CBP targeting to DNA or CBP activity. Fusions with the mixed lineage leukaemia/trithorax protein (MLL), monocytic leukaemia zinc finger (MOZ), MOZ related factor (MORF), and transcriptional intermediary factor (TIF) have all been shown to alter the HAT function of CBP [59].

There are many reasons to believe that dysregulation of HDAC functions is also involved in tumorigenesis. For example, HDACs may be recruited by certain

oncoproteins that subsequently modify HDACs subnuclear distribution and may ultimately lead to improper chromatin remodelling and gene expression. This possibility is illustrated by the fact that mutations or chromosomal translocations affecting the retinoic acid receptor dimers RAR/RXR may induce a persistent interaction between RAR/RXR and the SIN3-HDAC complex and hence promote leukaemia [59]. Additional support for a role of HDACs in tumorigenesis is demonstrated by the ability of HDAC inhibitors (including suberoyl anilide hydroxamic acid: SAHA, or trichostatin A: TSA) to induce growth arrest, differentiation, or apoptosis in a wide variety of human malignant cell lines *in vitro*, including bladder, breast, prostate, lung, ovary and colon cancer cells [74]. Cell growth arrest may be mediated by de novo expression of the cyclin dependent kinase inhibitor p21, as observed in the T24 human bladder carcinoma cells treated with SAHA [75]. HDAC inhibitors are also efficient to reduce tumour growth in chemically-induced animal models of mammary carcinoma, or following transplantation of prostate tumours or melanoma into nude mice [76, 77, 78].

2.4.4 DNA Methylation and Chromatin Remodelling in Normal and Cancerous Tissues

Another attractive aspect of the regulation of chromatin remodelling comes from recent data showing that SWI/SNF and HDACs interact with the DNA methylation machinery, and more particularly, that HDACs are involved in methylation-mediated aberrant gene silencing in cancer.

DNA methylation corresponds to the addition of a methyl group to DNA, most often onto a cytosine (Cp) followed by a guanosine (G). In promoter sequences of genes, CpG dinucleotides are clustered to form CpG islands. When methylated, CpG islands may trigger gene silencing. One of the most accepted explanations for promoter methylation-induced gene silencing is the recruitment of histone deacetylases by methyl-CpG binding proteins (MBPs) to methylated DNA that, in turn, causes deacetylation of adjacent histones and subsequent chromatin condensation [79]. Almost all MBPs interact with HDACs and require deacetylase activity to repress gene transcription (Figure 2.6). Methyl binding domain 2 (MBD2) related-protein is part of the histone deacetylase complex MeCP1 [80], while the presence of MBD1 in MeCP1 is still discussed [81, 82]. MBD3 is a component of the NuRD multisubunit assembly containing both ATP-dependent nucleosome remodelling activities, because of the presence of the SWI2/SNF2 ATPase Mi2, and histone deacetylase properties mediated by Sin3A, HDAC1, and HDAC2 [83]. MBD2 is not part of NuRD but can direct the complex to methylated DNA [83]. Transcriptional repression mediated by the methyl CpG-binding protein 2 (MeCP2) is partially dependent on histone deacetylase activity (mainly via Sin3A) [84]. In addition, interactions between HDAC1, HDAC2, and proteins responsible for DNA methylation, the DNA methyltransferases 1 (DNMT1) [85] and 3a [86] have been demonstrated to play a role in gene silencing. DNMT1 is usually located to replication foci where it methyl-

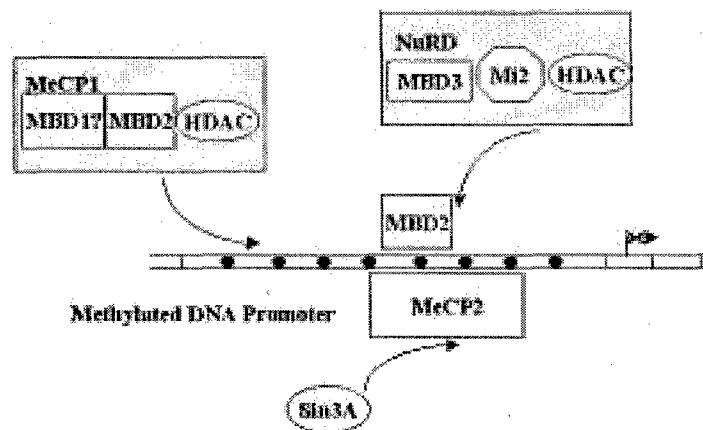


Figure 2.6

Methyl CpG binding proteins, chromatin remodelling factors, and methylated gene silencing. MBD2 is part of the histone deacetylase complex MeCP1 and binds to methylated DNA (black-filled circles), while the presence of MBD1 in MeCP1 is still being discussed. NuRD is composed of MBD3, Mi2-an ATP-dependent nucleosome remodelling factor- and histone deacetylases Sin3A, HDAC1, and HDAC2. MBD2 is not part of NuRD but directs the complex to methylated genes. MeCP2 binds to methylated DNA promoter sequences and can repress gene expression via HDAC (Sin3A) dependent or independent pathways.

lates newly synthesised DNA. The interaction between DNMT1 and HDAC2 at the level of replicating DNA during late S phase may further explain how a repressive heterochromatin state is maintained throughout cell generations [85]. Altogether, these observations connect chromatin remodelling, HDAC activity, and DNA methylation.

Aberrant DNA methylation is a hallmark of malignant cells. It may act upstream of histone modifications responsible for abnormal chromatin remodelling and improper gene expression by recruiting HDACs to sets of genes different from the ones targeted in a normal situation. In cancer, aberrant methylation associated with HDAC-mediated gene silencing has been shown for cell cycle genes (e.g., CDK2A and 2B) [87], genes involved in DNA repair (e.g., MLH1) [87], tumour suppressor genes (e.g., BRCA1) [88], inhibitors of invasion and metastasis (e.g., TIMP3) [87], and other key regulatory genes including the human telomerase reverse transcriptase [89] and RAR β [90].

2.4.5 Anti-Cancer Strategies Based on Chromatin Remodelling

The development of drugs targeting epigenetic mechanisms is relevant for cancer treatment because these drugs could influence the pattern of gene expression and thus cell behaviour. An approach based on the inhibition of HDACs is currently under investigation. The goal is to reinduce genes aberrantly silenced during tumorigenesis by acetylating histones. Inhibitors of HDACs have been shown to promote growth arrest and even differentiation or apoptosis in many tumour cell lines and in tumour bearing animals [74, 91]. The most studied HDAC inhibitors with potential therapeutic use are hydroxamic-acid-based hybrid polar compounds like SAHA; they act by blocking the catalytic site of HDACs. Several HDACs inhibitors are currently in phase I or II clinical trials and some objective tumour regressions have been observed [91, 92]. The biologic activity of HDAC inhibitors is monitored by measuring the increase in the level of acetylated histones in tumours. However, since these inhibitors are not specific of a particular deacetylase, we do not know whether the effect observed with tumour cells is solely due to histone hyperacetylation or whether treatment-induced acetylation of transcription factors, such as p53, might also regulate gene transcription and hence influence cell cycle and cell proliferation [93]. In addition, treatment-induced acetylation has also been detected in normal tissues, and we do not know the long-term consequences of hyperacetylation for these tissues. Results presented on chromatin remodelling during differentiation in part III of this chapter suggest caution should be taken when using HDAC inhibitors.

The regulation of gene expression by HDACs seems to be selective. Only about 2% of genes are affected upon treatment of normal and transformed cells with SAHA or TSA [74, 94]. For a number of genes this phenomenon may be due to the upstream regulation of gene expression resulting from DNA methylation. Indeed treatment with the HDACs inhibitor TSA is not sufficient to restore gene expression at certain loci, whereas treatment with the global demethylating agent 5 Aza 2'-deoxycytidine (5Aza) leads to a substantial reinduction of the expression of these genes, and additional treatment with TSA enhances the effect on 5Aza-mediated gene expression [87–90]. 5Aza has been used in clinical trials with various results; there were undesirable secondary effects (high toxicity, mucositis, diarrhoea, nausea, vomiting, and skin rash) and no significant antitumour activity could be measured for most types of cancer tested, including breast carcinoma [95, 96]. Additionally, preliminary results obtained in our laboratory showed that treatment of preformed breast tumour-like clusters with 5Aza did not alter malignant cell proliferation and did not induce glandular differentiation (unpublished results). Although combined treatment with 5Aza and HDACs inhibitors may be more efficient to restore the expression of methylated genes in cancer, it seems very dangerous to use such broad range inhibitors in patients. Therefore, knowing whether a specific type of acetylase and methyltransferase or MBP can be associated with the regulation of distinct subclasses of genes may be extremely useful for designing more specific, less toxic, and more efficient therapies.

2.5 Extracellular Matrix Signalling to the Cell Nucleus, Chromatin Remodelling, and Cell Behaviour

As we discussed in the first part of this chapter, the basement membrane plays a critical role in the induction and maintenance of epithelial cell differentiation. The formation of differentiated structures is accompanied by changes in gene expression and, according to recent data on chromatin remodelling reported in the second part of the chapter, it is expected that as a consequence of cell-basement membrane interaction, there will be significant alterations in the organisation of chromatin. Therefore understanding how basement membrane signalling regulates chromatin will be invaluable to identify intracellular pathways that may be modulated in future therapeutic strategies.

2.5.1 Chromatin Remodelling during Differentiation

A common feature for the study of the role of chromatin rearrangement in the differentiation process is to use TSA, a reversible inhibitor of HDACs. TSA treatment should maintain the expression of genes that would have been turned off normally by hypoacetylation during the differentiation process. Prevention of global histone hypoacetylation using HDAC inhibitors has various effects on cell differentiation according to the cell type. For instance, TSA treatment prevents the differentiation of nonmalignant rat stellate cells into myofibroblasts because, while TSA treatment hyperacetylates histones and should induce *de novo* gene expression, it also induces the inhibition of collagen I and III synthesis, as well as smooth muscle α -actin, a marker of differentiation [97]. Thus histone hypoacetylation somehow plays a role in differentiation. A possible explanation for the repressive effect on gene expression observed in the example described above is that TSA maintains the expression of certain genes, the products of which act as transcriptional repressors for other genes involved in the differentiation process.

As discussed in section 1.4, HDAC inhibitors may induce differentiation of tumour cells [91]. SAHA triggers differentiation in several transformed cell lines including the T24 human bladder carcinoma and MCF-7 human breast adenocarcinoma [74], indicating that hypoacetylation is involved in tumorigenesis. The differentiated phenotype was assessed according to morphological and proliferation parameters, as well as the expression of markers such as milk proteins in MCF-7 cells and gelsolin in T24 cells. However, it should be noted that the expression of differentiation markers is not necessarily indicative of true phenotypic reversion since this pattern of expression may also occur naturally in a number of tumours. Moreover, the effect of drug treatments on cell proliferation may be quite different between cells cultured as a monolayer (technique most often used) and cells cultured in conditions

that promote the formation of more physiologically relevant, tumour-like 3D multicellular structures. If indeed hypoacetylation plays a role in both differentiation and tumorigenesis, it is likely that the pattern of genes affected by this epigenetic modulation is different in each case. We do not know at this point if the genes affected by HDAC inhibitors in the examples described above are subject to methylation-mediated hypoacetylation or only methylation-free-hypoacetylation.

The use of 3D cultures in which cells are in contact with a reconstituted basement membrane has provided evidence that HDAC activity is involved in basement membrane-mediated mammary glandular differentiation [98, 99]. In the 3D culture system, mouse mammary epithelial cells differentiate into polarised glandular structures and express functional markers of milk production such as β -casein. Interestingly, glandular differentiation was accompanied by histone hypoacetylation, as shown for histone H4. When mammary epithelial cells were treated with TSA during the differentiation process, β -casein expression was prevented [99], indicating that shutting down certain genes is necessary for functional differentiation. Similarly in a human model of breast glandular differentiation, TSA-induced hyperacetylation triggered the loss of polarity, as shown by the fragmentation of the basement membrane, and pushed the cells back into the cell cycle [98]. Thus, chromatin remodelling mediated by histone deacetylation participates in basement membrane regulated events. At this time, there is no direct evidence showing that specific chromatin remodelling complexes are recruited by ECM-mediated differentiation. However, the potential connection between integrin signalling and HATs (e.g., CBP, SRC-1, and SRC-3), histone H3 kinases Rsk2 and Msk1, and histone H1 kinase cyclin E-cdk2 [58] described in the next paragraph, suggests that some of these chromatin remodelling factors might be involved in basement membrane-directed tissue differentiation.

2.5.2 Extracellular Matrix Signalling to Chromatin

A direct connection between basement membrane signalling and gene regulation was first described several years ago upon the discovery of ECM-responsive elements in several gene promoter sequences. A laminin responsive element was identified in the enhancer region of the β -casein gene [100] and, more recently, it was shown that the activation of this enhancer is modulated by the state of histone acetylation [101]. These results were obtained using mouse mammary epithelial cells stably transfected with the bovine β -casein promoter and subsequently treated with sodium butyrate, an inhibitor of HDACs. Sodium butyrate treatment was able to induce the transcription of β -casein controlled by the exogenous promoter, regardless of the presence of ECM. In another study, the expression of the $\alpha 5$ -integrin subunit was shown to be positively regulated by the ECM component fibronectin in rabbit corneal epithelial cells. Transcription factor Sp1 that binds to the fibronectin responsive element was involved in this regulation [102].

Conversely, the ECM may have a repressive action on gene transcription. This effect was demonstrated for the TGF- β gene promoter in mammary epithelial cells

[103]. When grown on plastic, mammary epithelial cells displayed a high level of TGF- β 1, while it was strongly downregulated in cells grown in the presence of reconstituted basement membrane. This observation was further confirmed using the chloramphenicol acetyl transferase (CAT) gene as reporter gene under the control of the TGF- β 1 promoter. CAT transcription was inhibited by the presence of the basement membrane.

Integrins are among the main transducers of signals induced by the ECM. Integrin binding to laminin activates a cascade of biochemical reactions as well as the reorganisation of the cytoskeleton and nuclear structure. Biochemical pathways include principally the tyrosine kinase cascade Ras/MAPK [104]. Interestingly Ras/MAPK signalling has been directly connected to chromatin remodelling complexes. Following a cascade of sequential activations by phosphorylation/dephosphorylation of Ras/Raf/MAPKK/MAPK, Erk MAPK is translocated into the nucleus where it interacts with chromatin remodelling factors. For instance, histone acetylases CBP, SRC-1, and SRC-3 are activated upon phosphorylation by Erk1 and Erk2. In addition, Erk can also activate histone H3 kinases, Rsk2 and Msk1, and histone H1 kinase cyclin E-cdk2 [58]. Therefore, interactions between integrins and basement membrane components may be able to direct chromatin remodelling, thereby controlling gene expression and cell phenotype.

Other proteins that may be involved in ECM or, more specifically, basement membrane signalling to chromatin could be found among proteins, other than histones, that contain a histone-fold sequence [105, 106]. These proteins can interact with other histone-fold bearing proteins and thus may regulate chromatin structure. Our laboratory is currently investigating this possibility with the example of the nuclear mitotic apparatus protein, NuMA. The nuclear distribution of NuMA is regulated by basement membrane-induced differentiation in mammary epithelial cells and seems to play a role in the control of MMP activity as well as basement membrane-mediated survival in these cells; NuMA distribution and function are altered in cancer [98, and unpublished results].

2.6 Conclusion

In this chapter, we have presented and discussed aspects of tumour biology research that have led scientists and clinicians to envision the development of novel anti-cancer strategies. These strategies illustrate an emerging view of cancer therapy, which aims at monitoring the illness chronically by influencing, and even re-programming, tumour cell behaviour rather than killing tumour cells. The goals are to prevent invasion and dissemination as well as restrain tumour cell proliferation. Unfortunately, the drugs that are currently being tested have a broad range of action and thus may also have effects on normal tissues. A better drug design will require at least,

- understanding the pathways that lead to the regulation of the expression of genes involved in the control of proliferation and differentiation from the cell membrane to the chromatin for each type of tissue, and
- undertaking pharmacological tests not only on tumours but also on nonmalignant and differentiated tissues.

The success of such difficult investigations will require thorough mathematical modelling of the different signalling networks and the dynamics of the multiprotein complexes involved.

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